Carbohydrate Polymers 86 (2011) 231–238

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis, characterization and biological safety of O-carboxymethyl chitosan used to treat Sarcoma 180 tumor

Meiling Zheng, Baoqin Han*, Yan Yang, Wanshun Liu

College of Marine Life Science, Ocean University of China, Qingdao 266003, PR China

ARTICLE INFO

Article history:
Received 14 January 2011
Received in revised form 13 April 2011
Accepted 16 April 2011
Available online 22 April 2011

Keywords:
Carboxymethyl chitosan
Sarcoma 180
Tumor
Biological safety
Biomaterial

ABSTRACT

Carboxymethyl chitosan (CM-chitosan) was prepared and characterized by FTIR and NMR spectroscopy, and its biological safety in tumor application was investigated both in vitro and in vivo. Cytotoxicity studies by MTT assay indicated that CM-chitosan was safe both on normal cell L02 and three tumor cell lines: Bel-7402, SGC-7901 and Hela in vitro. CM-chitosan also improved the TGF-α secretion of L02 cell ($P < 0.05$), whereas decreased levels of TGF-α and VEGF secreted by Bel-7402 cell ($P < 0.05$), which are compatible with the observations at cell levels. In vivo, transplant tumor model of sarcoma 180 was established in mice and CM-chitosan was administered through intraperitoneal injection. Experimental data indicated that CM-chitosan was safe in vivo and slightly inhibited growth of sarcoma 180 and enhanced body immunity via elevation of serum IL-2 and TNF-α levels in treated mice ($P < 0.05$). These results suggest that CM-chitosan is safe in tumor application as biomedical material.

1. Introduction

Chitosan is being used in tissue engineering because of its ideal biocompatibility (Abdull Rasad, Halim, Hashim, Rashid, Yusof, & Shamsuddin, 2010), biodegradability (Jolles & Muzzarelli, 1999), mucoadhesivity (Mori et al., 1997) and its non-toxic, non-immunogenic and non-carcinogenic degradation products (Aiedehe, Gianasii, Orienti, & Zecchi, 1997; Muzzarelli, 2010; Shigemasa & Minami, 1996). Muzzarelli et al. improved the water solubility of chitosan by reacting it with glyoxylic acid to yield NCM-chitosan (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982).

Carboxymethyl chitosan (CM-chitosan) is a water soluble chitosan derivative by introducing the CH_2–COOH function into chitosan and endow it with some outstanding biological properties. Previous studies demonstrated that CM-chitosan could promote the proliferation of the normal skin fibroblast, inhibit the proliferation of keloid fibroblast, stimulate the extracellular lysozyme (Chen, Wang, Liu, & Park, 2002; Zhu & Fang, 2005) and promote osteogenesis (Muzzarelli, 2009; Muzzarelli, Ramos, & Stanic, 1998). As versatile biomedical material, CM-chitosan always conjugated or entrapped or self-assembled with agent (Mathew, Mohan, Manzoor, Nair, Tamura, & Jayakumar, 2010; Xia, Wang, Nie, Peng, & Guan, 2005) and extensively applied in tumor therapy research (Anitha et al., 2010). However, safety evaluation of CM-chitosan has been focused on the compounds and in vitro model (El-Sherbiny, 2009; Wang, Chen, Zhong, & Xu, 2007), little reported work of CM-chitosan safety evaluation both in vitro and vivo has been discussed, especially in tumor therapy. Moreover, the actual metabolic pathway and degradation products of CM-chitosan in the body have been as yet unknown. Therefore, additional biological safety research of CM-chitosan in tumor application is necessary.

The scope of this study was to prepare and characterize OCM-chitosan, to investigate its cytotoxicity by using normal cell and three tumor cells in vitro, and the influence of CM-chitosan on the growth of sarcoma 180 and immune response of tumor-bearing mice were also investigated, providing experimental basis for CM-chitosan utilization in tumor application as biomedical material.

2. Experimental

2.1. Materials

Chitosan (degree of deacetylation = 78.0%) was supplied by Qingdao Biotemed Biomaterial Co., Ltd (China). RPMI 1640 media and Newborn calf serum (NCS) were obtained from GIBCO (USA). Human transforming growth factor-α (TGF-α) ELISA kit, human vascular endothelial growth factor (VEGF) ELISA kit, mouse interleukin-2 (IL-2) ELISA kit and mouse tumor necrosis factor-α (TNF-α) ELISA kit were all procured from ShangHai JinMa Experimental Equipment Co., Ltd. (China). Other chemicals were of

Abbreviations: Bel-7402, human hepatoma cell; BWC, body weight change; CM-chitosan, carboxymethyl chitosan; DS, degree of substitution; NCM-chitosan, N substituted carboxymethyl chitosan; OCM-chitosan, O substituted carboxymethyl chitosan.

* Corresponding author. Current address: Room 220, Science Building, 5# Yushan Rd., Qingdao, China. Tel.: +86 532 8203 2105; fax: +86 532 8203 2105.
E-mail address: baoqinh@ouc.edu.cn (B. Han).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.04.038

reagent grade. Human normal liver cell L02, human hepatoma cell Bel-7402, human gastric cancer cell SGC-7901 and human cervical carcinoma cell Hela were all obtained from the Institute of Pharmacology of Ocean University of China and cultured in RPMI 1640 medium supplemented with 10% NCS, 100 U/L streptomycin and 100 U/L penicillin in an incubator set at 37 °C, 5% CO_2 and 95% humidity. Sarcoma-180-bearing mouse was obtained from Jinan DingGuo Biotech.Co. Ltd. (China). Kunming mice (half female and half male), 35 days old, weighing 20 ± 1 g, were obtained from Qingdao Experimental Animal Center (China) and treated according to the standards supported by the animal protection committee of China.

2.2. Synthesis and purification of CM-chitosan

CM-chitosan was prepared from chitosan as described previously (Bidgoli, Zamani, & Taherzadeh, 2010; Chen & Park, 2003; Zamani, Henriksson, & Taherzadeh, 2010) with minor modifications. The brief processes and the methods of CM-chitosan preparation were shown in Fig. 1. Chitosan (20 g), sodium hydroxide (27 g) and solvent (200 ml) were added into a flask to swell and alkalize at −20 °C for 24 h. The monochloroacetic acid (30 g) was dissolved in isopropanol (40 ml), then added into the reaction mixture dropwise for 30 min and reacted at 50 °C for 7 h. The reaction was then stopped by adding 70% ethanol to the mixture and CM-chitosan was separated by filtration. It was rinsed by 70% ethanol for five times and dehydrated with absolute alcohol. This primary product was the sodium salt of CM-chitosan. The sodium salt of CM-chitosan was converted to CM-chitosan by immersing in 70% ethanol and adding 32% HCl. Then, the resultant suspension was mixed for 30 min and filtered. The sample was dissolved in deionized, distilled H_2O and dialyzed for 3 days to remove impurity. Finally, CM-chitosan sample was vacuum freeze dried and stored desiccated until use.

2.3. Characterization of CM-chitosan

The IR spectra of chitosan and the sodium salt of CM-chitosan were recorded on an FT/IR-430 Fourier Transform Infrared Spectrometer. Pellets were formed from 2 mg sample and 100 mg of KBr. Data analysis was carried out using Jwstda-32 (Sashiwa, Saimoto, Shigemasa, Ogawa, & Tokura, 1990; Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996).

^1H and ^{13}C NMR spectra of chitosan and CM-chitosan were recorded on a Bruker DPX300 spectrometer using acetone as internal standard and 1% (v/v) DCl D_2O solution and D_2O as solvent, respectively, at 25 °C.

The molecular weight (MW) of CM-chitosan was measured with gel permeation chromatography using high performance liquid chromatograph, chromatogram column TSK G4000PWXL, flowing phase was 0.02 M phosphate buffer, flowing rate was 0.8 ml/min. Data were integrated and analyzed using the GPCW32. Degree of substitution (DS), defined as the number of carboxymethyl groups per 100 glucosamine groups of chitosan, was determined by alkalimetry (Wan, Khor, Wong, & Hastings, 1996). The content of free amino defined as the average number of free amino binding to N atom of per 100 glucosamine groups of CM-chitosan was determined by potentiometric (Muzzarelli, Tanfani, & Emanuelli, 1984).

2.4. In vitro evaluation of toxicity

Cell cytotoxicity assays are amongst the most common in vitro bioassay methods used to predict the toxicity of substances in various tissues (Lee, Kim, Kim, & Kim, 2000; Sanchez, Mitjans, Infante, & Vinardell, 2004). In this study, three tumor cell lines and human normal liver cell L02 were used to evaluate the cytotoxicity of CM-chitosan by MTT assay (Paolino, Muzzalupo, Ricciardi, Celia, Picci, & Fresta, 2007). Cells at the logarithmic growth phase were seeded at a density of 3×10^4 cells/ml in a 96-well culture plates in a total volume of 200 μl per well and incubated for 24 h at 37 °C and 5% CO_2 to allow the adhesion of culture cells. Then, the culture medium was aspirated and replaced with fresh medium supplemented with corresponding concentrations of CM-chitosan, respectively. Following incubation for 2 days and 4 days, 20 μl MTT solution (5 mg/ml dissolved in PBS buffer) were added to each well. The plate was re-incubated for 4 h and 150 μl DMSO was introduced to dissolve the insoluble blue formazan precipitate produced by MTT reduction. Then the plate was shaken for 3 min and the optical density (OD) was measured at 492 nm with an enzyme immunoassay instrument (Multiskan MK3, Thermo Labsystems, USA). The cytotoxicity of CM-chitosan was expressed as the percentage reduction of cell viability in terms of relative proliferation ratio (RGR) and calculated with the following formula: $RGR = (OD_1 - OD_0)/(OD_2 - OD_0) \times 100\%$, where OD_0, OD_1 and OD_2 were the average OD of the medium, treated and control groups, respectively.

2.5. ELISA for TGF-α and VEGF proteins

L02 cells and Bel-7402 cells at the logarithmic growth phase were seeded into a 96-well plate in the density of 4×10^4 per well and incubated for 24 h. Then, solutions were added to the cells containing serum-free RPMI 1640 containing varying concentrations of CM-chitosan. The culture supernatants of cells were collected after 24 h, centrifuged at 2500 rpm for 20 min to measure the levels of TGF-α and VEGF by ELISA using commercially available assay systems according to the manufacturer's instructions.

2.6. In vivo evaluation of CM-chitosan on the solid tumor

To observe the therapeutic response of CM-chitosan in tumor application, the effectiveness of CM-chitosan on the sarcoma-180-bearing mice were evaluated (Xu, Bian, & Chen, 1991). Sarcoma-180-bearing mice were prepared by subcutaneous transplantation of 2×10^6 six-day-old Sarcoma 180 ascites tumor cells into the right oxter region of mice on day 0. Twenty-four hours after inoculation, mice were randomly divided into four groups. CM-chitosan solutions were administered to the treated group in the dose of 75, 150 and 300 mg/kg body weight through intraperitoneal injection every other day for 12 days. The control group received 0.9% normal saline on the same schedule. The mice in different groups were sacrificed on day 13. Subsequently, body weight change (BWC) was measured at the start and at the last day of treatment to investigate the long-term physical toxicity of CM-chitosan. The tumors were weighed to evaluate the effect of CM-chitosan on the tumor growth. Immune organs including spleen, thymus and liver of the mice were also removed and weighed to obtain the index of the spleen, thymus and liver. Spleen index (mg/g) = spleen weight/body weight, thymus index (mg/g) = thymus weight/body weight, Liver index (mg/g) = liver weight/body weight.

2.7. Effects of CM-chitosan on serum IL-2 and TNF-α levels

Forty sarcoma 180-bearing mice were randomly assigned into four groups, control group and CM-chitosan (75, 150 and 300 mg/kg) groups. After CM-chitosan administration through intraperitoneal injection every other day for 12 days, blood was collected on day 13 and maintained at room temperature for 4 h with natural coagulation, followed by centrifugation at 1500 rpm, and the supernatants were used for ELISA termination of IL-2 and TNF-α levels.