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

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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.

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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.

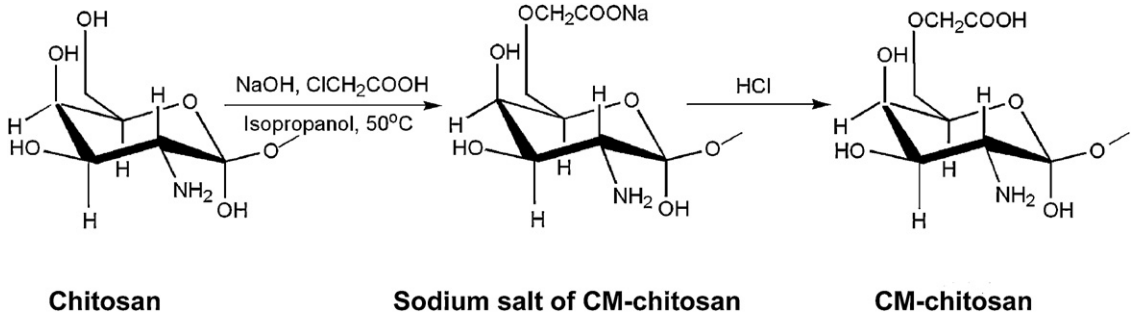

Fig. 1. Synthesis of CM-chitosan from chitosan.

Table 1
Physicochemical property of CM-chitosan sample.

Sample	MW (kDa)	DS (%)	Content of free amino (%)
CM-chitosan	340.0	72.7	76.8

2.8. Statistical analysis

Data were indicated as means ± SD. The data from each experiment was analyzed by one-way ANOVA. Values of $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Preparation and properties analysis of CM-chitosan

Carboxymethylation of chitosan was achieved with monochloroacetic acid and sodium hydroxide. The prepared CM-chitosan sample had good water solubility. MW, DS and content of free amino of CM-chitosan were important parameters, which could influence its performance in many of its applications and they were given in Table 1. The MW of CM-chitosan sample was 340.0 kDa which was extensively used in biomedical applications. DS of CM-chitosan was 72.7%, indicating that 72.7 carboxymethyl groups were introduced to per 100 glucosamine units of chitosan. The content of free amino of CM-chitosan was 76.8% revealed that there was survival of the N-acetylglucosamine units in CM-chitosan molecule.

3.2. Structure and chemical composition

The chemical structure of original chitosan and products were confirmed by IR, ^{1}H and ^{13}C NMR spectroscopy.

3.2.1. FT/IR data analysis

The IR spectra of sodium salt of CM-chitosan and chitosan are shown in Fig. 2. Fig. 2B showed the basic characteristics of chitosan at: 1094 cm^{-1} (C–O stretch), 1153 cm^{-1} (bridge-O stretch), 1556 cm^{-1} (N–H bend), 2881 cm^{-1} (C–H stretch), and 3421 cm^{-1} (O–H stretch) (Brugnerotto, Lizardi, Goycoolea, Arguelles-Monal, Desbrieres, & Rinaudo, 2001). In the IR spectrum of the sodium salt of CM-chitosan given in Fig. 2A, the new absorption peaks at 1407 cm^{-1} and 1598 cm^{-1} could be, respectively, assigned to the symmetry and asymmetry stretch vibration of COO^-. Additionally, the formation of the sodium salt of CM-chitosan was also confirmed by the intensification of the band at 1068 cm^{-1} corresponding to C–O–C stretching. The C–O stretching band at 1030 cm^{-1} corresponding to the primary hydroxyl group disappears, verifying a high carboxymethylation of OH-6 (Zhang, Guo, Zhou, Yang, & Du, 2000; Zhao, Kato, Fukumoto, & Nakamae, 2001). Therefore, the results of the IR spectroscopy confirmed carboxymethylation of chitosan.

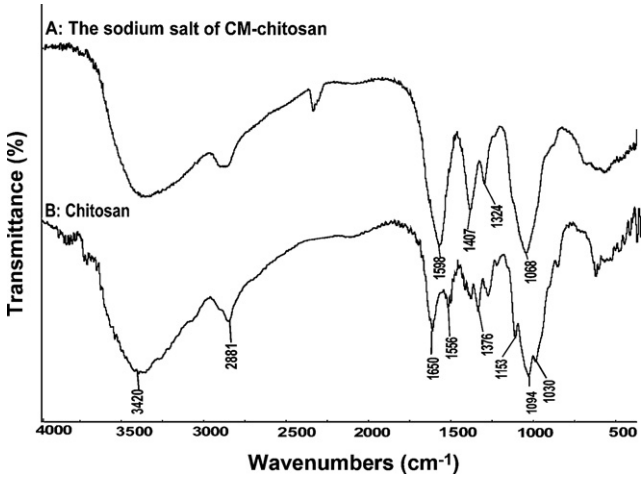

Fig. 2. FTIR spectra of CM-chitosan (A) and chitosan (B).

3.2.2. NMR data analysis

Fig. 3 presents the ^{1}HNMR spectra of chitosan and CM-chitosan. The proton assignment of chitosan (Fig. 3B) was as follows (ppm): 1.91 (CH_3, acetamido group of chitosan), 3.04 (CH, carbon 2 of glucosamine ring), 3.61 (CH, carbon 2 of glucosamine ring with the substituted amino group), 3.7–3.9 (CH, carbon 3, 4, 5 and 6 of glucosamine ring), 4.75 (CH, carbon 1 of glucosamine ring) (Kubota & Eguchi, 1997). According to what has been previously reported (Chen & Park, 2003), the characteristic proton signals of CM-chitosan (Fig. 3A) appeared in the range of 4.0–4.1 ppm, indicating that carboxymethyl groups linked to chitosan.

Fig. 4 depicts the ^{13}C NMR spectra of CM-chitosan and chitosan. The peaks of chitosan (Fig. 4B) at $\delta = 101.7$, 61.7, 70.3, 77.6, 75.4 and 57.2 ppm were attributed to the C-1, C-2, C-3, C-4, C-5 and C-6, respectively (Sun, Du, Fan, Chen, & Yang, 2006). For the CM-chitosan described in Fig. 4A, signals at 55.2 and 178.9 ppm were, respectively, assigned to carbonyl carbon O–CH_2 and COOH carboxyl group, verifying a high carboxymethylation of OH-6. The N-carboxymethyl substituent is not present because of the absence of peaks at 47 and 168 ppm for N–CH_2 and COOH, respectively. The results were consistent with the reported spectra (El. Shafei, & Abou-Okeil, 2011; Zhao, Wang, Ye, & Wang, 2002).

3.3. Effects of CM-chitosan on the growth of four cells in vitro

The determination of cell viability is a mean of observing the in vitro cytotoxicity of biomaterials through detrimental intracellular effects on mitochondria and metabolic activity. Fig. 5 presents

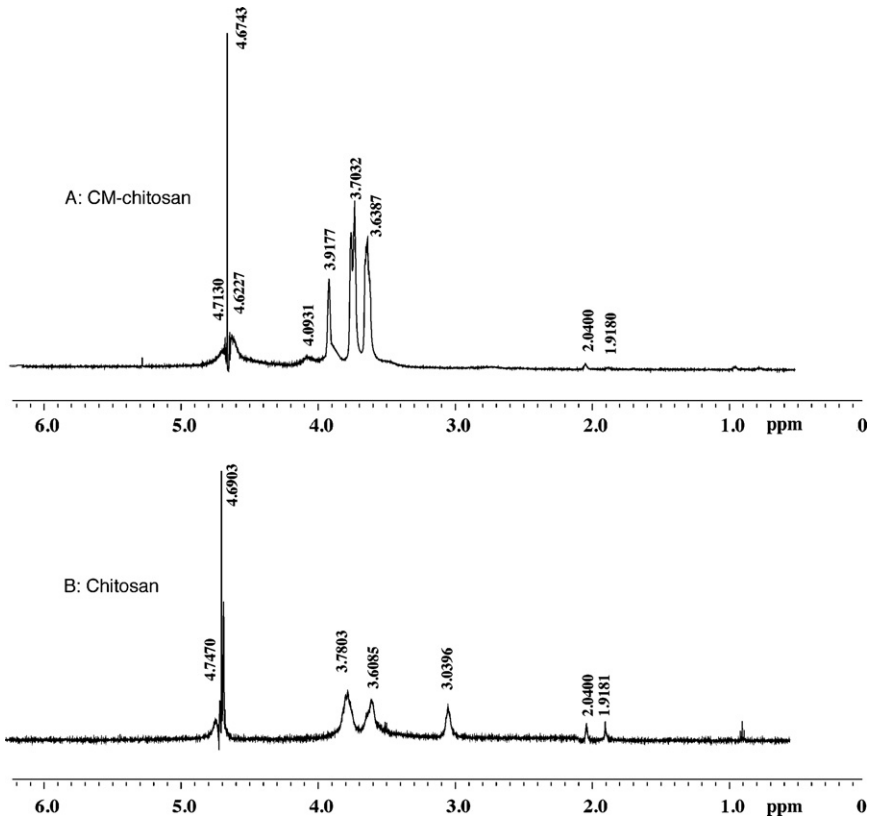

Fig. 3. ^{1}H NMR spectra of CM-chitosan (A) and chitosan (B).

the result of four cells growth influenced by CM-chitosan. The results showed there was no reduction of L02 cells on treatment with CM-chitosan for all tested concentration, it was clear that the CM-chitosan was nontoxic to L02 cells. With regard to tumor cell models, CM-chitosan exerted slight growth inhibitory effects on the Bel-7402, SGC-7901 and Hela cells within the concentration of 50–5000 μg/ml treated for 2 days ($P < 0.05$), whereas the inhibitory effect were attenuated on day 4. Since the RGR reduction of tumor cells were less than 20% for all tested concentration, CM-chitosan was still safe to tumor cells.

According to these results, it was clear that CM-chitosan was safe in tumor application at cellular level in vitro. Interestingly, CM-chitosan exerted differential cytotoxicity on tumor cells and normal cells. This may be due to morphologic and physiologic differences between normal and tumor cells caused by gene alteration during cancer formation, such as enhancement of membrane permeability,

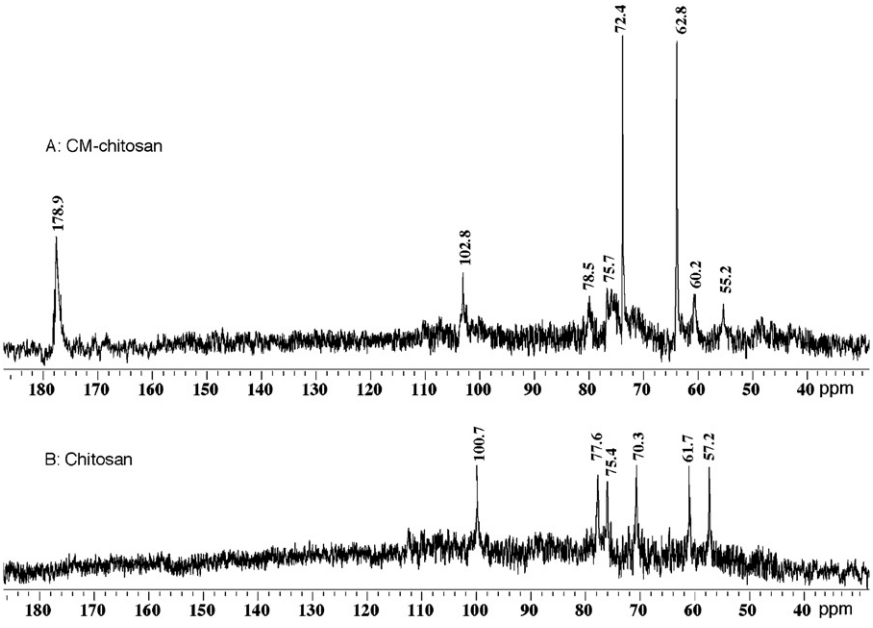

Fig. 4. ^{13}C NMR spectra of CM-chitosan (A) and chitosan (B).

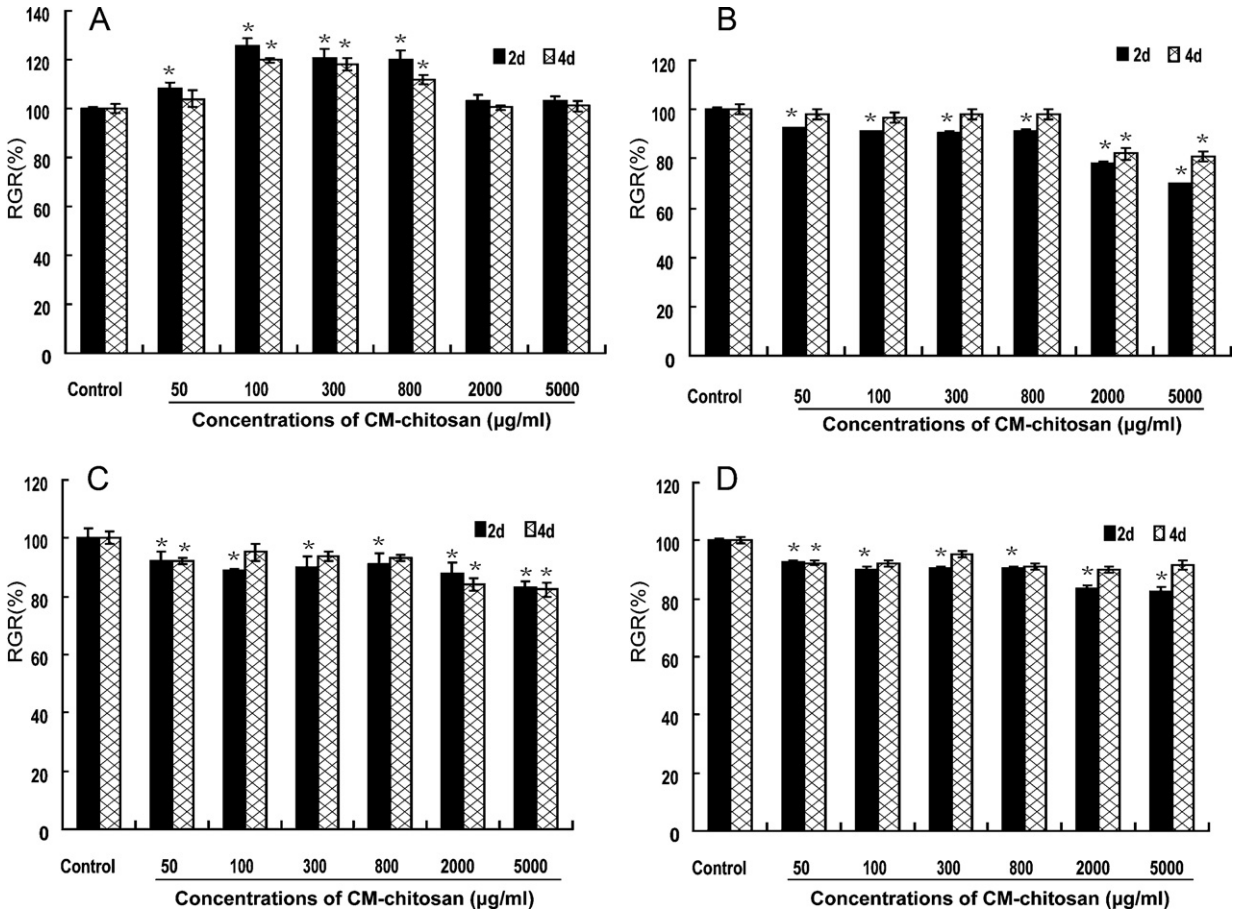

Fig. 5. Effects of CM-chitosan on the proliferation of four cells, $n = 5$, Means ± SD,*$P < 0.05$ vs. control group. (A) Human normal liver cell L02; (B) human hepatoma cell Bel-7402; (C) human gastric cancer cell SGC-7901; (D) human cervical carcinoma cell Hela. RGR was the relative cell proliferation ratio and was 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.

altered membrane signaling proteins, an increase in the velocity of RNA and DNA production and increased flow of metabolites in the oxidative phosphorylation pathway and so on (Ertel, Verghese, Byers, Ochs, & Tozeren, 2006).

3.4. Effect of CM-chitosan on the TGF-α and VEGF secretion

To determine the underlying mechanism of twofold effects of CM-chitosan on the proliferation of normal human liver cell L02 and human hepatoma cell Bel-7402, relevant cytokines secreted by them were measured in this study. TGF-α was the only autocrine growth factor involved liver regeneration and liver cell growth secreted by L02 cell. The high levels of TGF-α expression always accompanied with growth promotion of liver cells. VEGF is a chemical signal produced by vascular endothelial cells and tumor cells, whose level is positively correlated with angiogenesis, tumor metastasis and growth promotion of tumor cells. Fig. 6 displayed the effects of CM-chitosan on the TGF-α and VEGF protein secretion of Bel-7402 cells and TGF-α protein secretion of L02 cells at 24 h. CM-chitosan dramatically increased the TGF-α secretion of

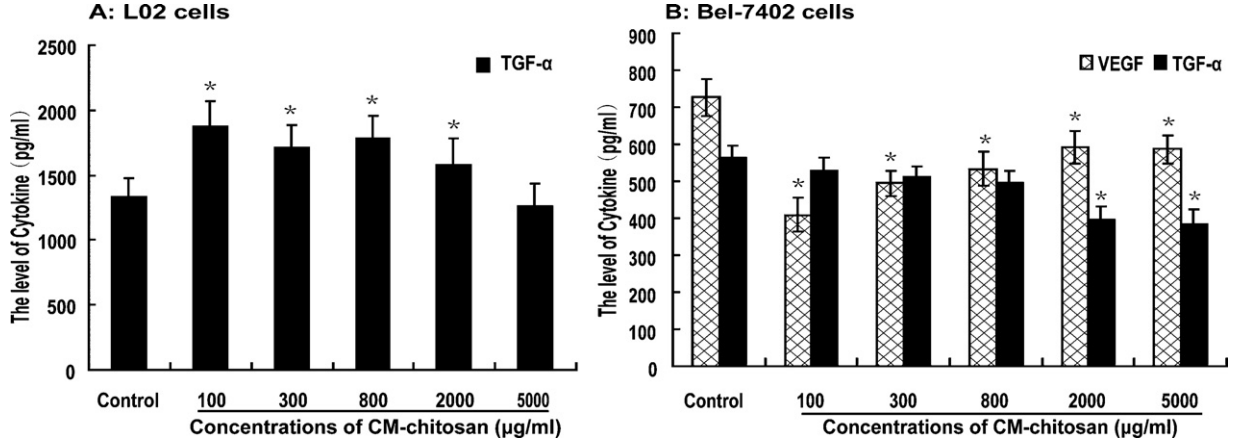

Fig. 6. Effects of CM-chitosan on the levels of cytokines secreted by L02 cells and Bel-7402 cells at 24 h, $n = 5$, Means ± SD,*$P < 0.05$ vs control group.

Table 2
Effect of CM-chitosan on the growth of sarcoma 180 (means ± SD, $n = 10$).

Group	Dose (mg/kg)	BWC[a] (g)	Tumor weight (g)	Thymus index (mg/g)	Spleen index (mg/g)	Liver index (mg/g)
Control	0	11.93 ± 0.81	2.38 ± 0.75	6.66 ± 1.88	11.21 ± 2.89	64.32 ± 9.89
CM-chitosan	75	11.48 ± 1.06	1.68 ± 0.61[b]	6.51 ± 2.30	11.18 ± 1.51	65.21 ± 8.67
CM-chitosan	150	12.37 ± 1.16	1.58 ± 0.71[b]	6.98 ± 0.98	13.83 ± 2.36[b]	65.07 ± 8.01
CM-chitosan	300	11.34 ± 1.44	1.43 ± 0.51[b]	7.71 ± 2.45[b]	15.61 ± 2.23[b]	65.44 ± 7.87

[a] BWC was the body weight difference between the start and the last day of treatment to investigate the long-term physical toxicity of CM-chitosan.
[b] $P < 0.05$ vs control group.

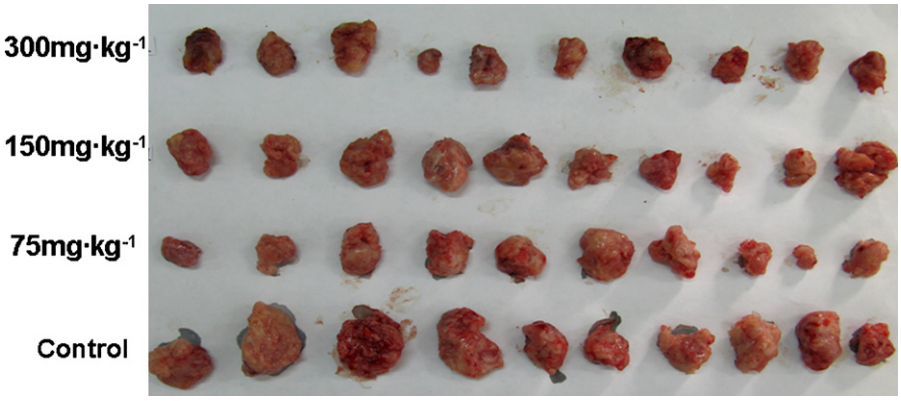

Fig. 7. Effects of CM-chitosan on the sarcoma 180 morphology of each group.

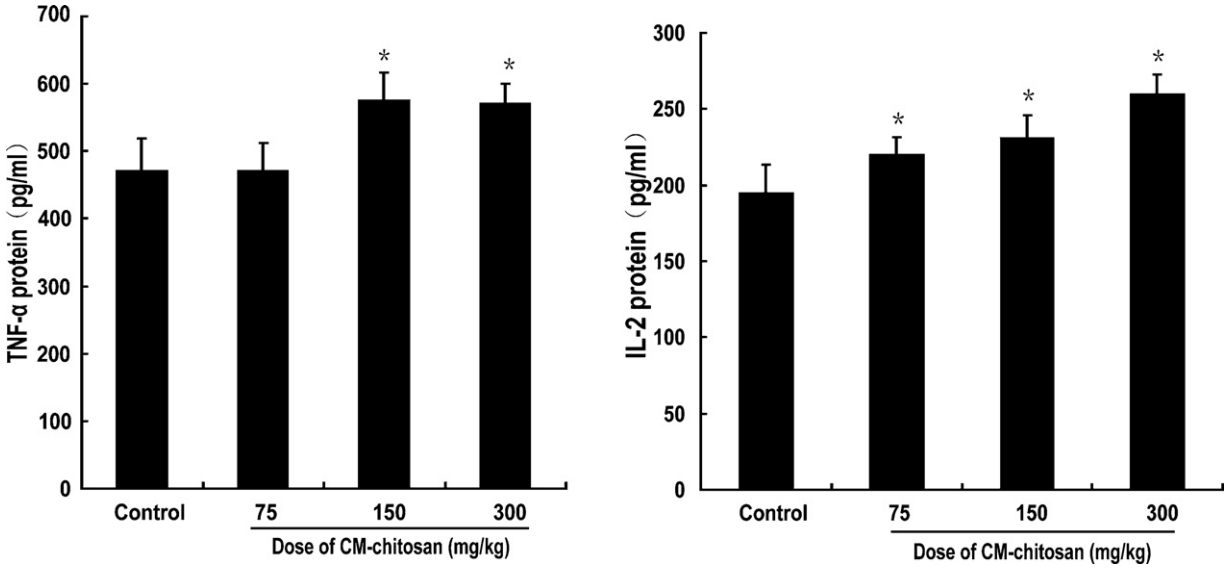

Fig. 8. CM-chitosan elevated the serum levels of IL-2 and TNF-α in sarcoma 180 mice, $n = 10$, means ± SD, $^*P < 0.05$ vs control group.

L02 cells for all tested concentration with except to 5000 μg/ml ($P < 0.05$) whereas there was no significant effect to TGF-α secretion of Bel-7402 cells within the same CM-chitosan concentration. With regard to VEGF, CM-chitosan inhibited the VEGF secretion of Bel-7402 cells for all tested concentration. On the contrary, L02 cells scarcely secreted VEGF and the concentrations of VEGF secreted by L02 cells were all lower than 100 pg/ml in this study and no significant difference was observed for all tested concentration.

The cytokine levels results obtained herein are consistent with the previous observations at cell levels. This may account for the twofold bioactivities of CM-chitosan on the growth of L02 and Bel-7402 cells.

3.5. Effects of CM-chitosan on the growth of solid tumors in mice

Different administration of materials may exert different in vivo toxicity (Lanigan & Yamarik, 2002). CM-chitosan applied in tumor therapy as biomedical material were simulated following intraperitoneal administration. As shown in Table 2, there were not obvious differences of measured parameters including body weight change, liver index as well as thymus index and spleen index in low dosage between control and CM-chitosan treated group. Only a slight tumor weight loss can be observed for the entire CM-chitosan treated group. However, mice treated with CM-chitosan did not show any sign of apparent toxicity or body weight loss compared with controls.

The real role of CM-chitosan in the body of tumor-bearing mice has remained unknown. However, previous study found that CM-chitosan was rapidly distributed to blood and many organs including liver, spleen, and kidney after intraperitoneal administration to rats, especially for relatively high molecular weight (~300 kDa) forms (Dong et al., 2010). Spleen, thymus and liver are important parts of the immune system. In this study, CM-chitosan was safe to body except for slight anticancer activity against sar-

coma 180 in mice which may be due to an immuno-enhancing effect of CM-chitosan. The morphological difference of sarcoma 180 tumors affected by CM-chitosan is shown in Fig. 7.

3.6. The up-regulation of immune response induced by CM-chitosan

To better understand the slight anticancer effect of CM-chitosan and body immune response induced by CM-chitosan, serum IL-2 and TNF-α level of sarcoma-180-bearing mice were investigated. IL-2 is an autocrine growth factor for T cells (Taniguchi & Minami, 1993), which also activates other cells including B cells (Waldmann et al., 1984) and natural killer cells (Henney, Kuribayashi, Kern, & Gillis, 1981), is pivotal for the generation and regulation of the immune response. TNF-α which is predominantly biosynthesized by cells of the monocytic lineage is a potent immunomodulator and proinflammatory cytokine that has been implicated in the pathogenesis of autoimmune and infectious diseases (Wilson, Symons, McDowell, McDevitt, & Duff, 1997). As shown in Fig. 8, the serum concentrations of IL-2 and TNF-α of CM-chitosan treated group were higher than that of control group, especially in the dose of 150 mg/kg and 300 mg/kg and similar for IL-2 at 75 mg/kg ($P < 0.05$), suggesting that CM-chitosan could enhance body immune function in the certain dose and this result would be further proved by the increase of spleen index and thymus index in high dose CM-chitosan treated groups (Table 2).Conclusions

In this study, 340 kDa OCM-chitosan which was widely used in biomedical applications was synthesized, characterized and its biological safety in tumor therapy was systematically investigated both in vitro and in vivo for the first time. In vitro, CM-chitosan was safe in the culture of human normal liver cell L02, human hepatoma cell Bel-7402, human gastric cancer cell SGC-7901 and human cervical carcinoma cell Hela. Results at cytokine levels were compatible with the observations at liver cell levels. In vivo, it was observed that CM-chitosan was nontoxic to body and enhanced body immune response via elevation of spleen index, thymus index and serum levels of IL-2 and TNF-α in sarcoma-180-bearing mouse in certain dose. In summary, it is clear that CM-chitosan is suitable biomaterials for preclinical and clinical use in tumor therapy.

Acknowledgements

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