Carbohydrate Polymers 125 (2015) 53–60

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Preparation and anti-tumor metastasis of carboxymethyl chitosan

Zhiwen Jiang, Baoqin Han*, Hui Li, Xiuhua Li, Yan Yang, Wanshun Liu

Biochemistry Laboratory, College of Marine Life Sciences, Ocean University of China, 5 No. Yushan Road, Qingdao 266003, PR China

ARTICLE INFO

Article history:
Received 1 October 2014
Received in revised form 12 February 2015
Accepted 14 February 2015
Available online 26 February 2015

Keywords:
Carboxymethyl chitosan
Hepatic
Anti-tumor
Metastasis

ABSTRACT

Carboxymethyl chitosan (CMCS), one of the most important water soluble chitosan derivatives, has great potentials in biomedical applications due to its excellent water solubility, biodegradability, biocompatibility, and non-toxicity. In the present study, the anti-tumor metastasis effect of CMCS on hepatic tumors was evaluated using human hepatic cancer cell BEL-7402 and mouse hepatoma 22 cells. The results suggested that CMCS could significantly inhibit tumor cell migration *in vitro*, and reduce the expression of matrix metalloproteinase-9 in BEL-7402 cells in a dose-dependent manner ($P < 0.05$). Furthermore, CMCS significantly inhibited the lung metastasis of hepatoma-22 in Kunming mice ($P < 0.05$). Significant improvement of the lung injury caused by the metastasis of H22 was also observed. The results suggested that the inhibitory effect of CMCS could be attributed in part to the decreased levels of vascular endothelial growth factor and E-selectin in CMCS treated mice.

1. Introduction

Hepatocellular carcinoma is a major cause of cancer death worldwide (Takigawa & Brown, 2008), and it has been a major public health problem in developing countries which accounts for 80% of deaths in the world (Chen & Zhang, 2011). Liver metastasis, accounting for one-third of all tumor metastasis, is the most difficult to treat in the clinic and usually causes the death of liver cancer patients (Palomares, Garcia-Alonso, San, Mendez, & Alonso-Varona, 2014). Metastases arise when tumor cells break away from a primary tumor to establish a secondary tumor in a distant site (Fidler, 2003; Weiss, 2000; Geiger & Peeper, 2009). Metastasis is a multistep process, which includes penetrating the walls of lymphatic and/or blood vessels, infiltrating into the circulation system, re-penetrating through the vessels, docking and proliferating in the distant organs to form a metastatic tumor (Li et al., 2013). Interference with any step would lead to the blockade of the entire metastatic process. Tumor metastases are mainly divided into three categories: lymphatic metastasis, blood–borne metastasis and planting metastases.

Carboxymethyl chitosan (CMCS), a water soluble derivative of chitosan, with enhanced physicochemical and biological properties compared to chitosan (Upadhyaya, Singh, Agarwal, & Tewari, 2013), has wide applications in cosmetic, food, pharmaceutical, and biomedical materials due to its excellent water solubility, and film forming as well as moisture retention ability. CMCS has been used as biomaterials in various research and applications, such as wound healing (Wang, Lu, Ao, Gong, & Zhang, 2010; Lu et al., 2007), tissue engineering (Budiraharjo, Neoh, & Kang, 2012; Tang, Sun, Fan, & Zhang, 2012), drug delivery (D'Agostini-Junior, Petkowicz, Couto, de Andrade, & Freitas, 2011; Yu et al., 2012; Zheng et al., 2011a; Zou et al., 2012), gene therapy (Zhang et al., 2009; Li et al., 2012) and bioimaging (Shi et al., 2009; Bhattacharya et al., 2011). In our previous studies, the anti-tumor activity of CMCS was investigated both *in vitro* and *in vivo*. CMCS showed cytotoxicity on certain tumor cells such as HeLa, SGC-7901 and Bel-7402 *in vitro* and slightly inhibited the growth of sarcoma 180 and enhanced body immunity *in vivo* (Zheng, Han, Yang, & Liu, 2011b). In the present study, the anti-metastatic effect of CMCS was assessed *in vitro* and *in vivo*, and our results provided experimental data for the utilization of CMCS in liver tumor surgery and anti- hepatic tumor drug targeting.

2. Experimental

2.1. Reagents and antibodies

CMCS was synthesized, purified and characterized by our laboratory. Rabbit polyclonal antibody against mouse matrix metalloproteinase-9 (MMP-9), Horse Radish Peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody, 3,3-diaminobenzidine (DAB), Mouse vascular endothelial growth factor (VEGF) ELISA Kit and Mouse E-Selectin ELISA Kit were from

* Corresponding author. Tel.: +86 532 82032105; fax: +86 532 82032105.
E-mail address: baoqinh@ouc.edu.cn (B. Han).

http://dx.doi.org/10.1016/j.carbpol.2015.02.039
0144-8617/

Boster Biological Engineering Co., Ltd (Wuhan, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium and fetal bovine serum (FBS) was from GIBCO BRL (Grand Island, NY, USA). 96 well plates, 24 well plates and transwell plates were from Corning INC. (USA). Shodex STANDARD P-82 Pullulan was from Showa Denko (Tokyo, Japan).

2.2. Animals and cell lines

Kunming mice (half male and female) weighing 18–22 g were supplied by institute of drug inspection of Qingdao City, China. All animals were kept under a 12 h light-dark cycle at consistent temperature (25 ± 3 °C) and relative humidity (60–70%). Experiments were performed in accordance with the ethical guidelines of the Shandong Province Experimental Animal Management Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The human hepatocellular liver carcinoma cell line BEL-7402 and mouse hepatoma 22 cells were supplied by OUC Institute of Materia Medica. Both cells were grown and subcultured at 37 °C in a humidified, 5% CO_2 incubator in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells in exponential growth were used in all experiments. Cell viability, as determined by trypan blue exclusion, ranged from 95% to 98%.

2.3. Synthesis and properties analysis

CMCS was synthesized and purified in the laboratory as previously reported (Zheng et al., 2011b). Carboxyl structures and molecular weight (MW) was determined by fourier-transformed infrared spectroscopy (FTIR) and high performance liquid chromatography (HPLC). Acid-base titration method was used to determine the degrees of substitution (DS) and degree of deacetylation (DD). Heavy Metal Content (HMC) and Intracellular Toxin Content (ITC) were measured strictly in accordance with the national standard of China.

2.4. In vitro 2-dimensional migration assay

CMCS was dissolved in complete medium containing 10% FBS at concentrations of 5.0 mg/ml, 4.0 mg/ml, 3.0 mg/ml, 2.0 mg/ml and 1.0 mg/ml. BEL-7402 cells in logarithmic growth phase were trypsinized and resuspended with complete medium at a concentration of 2.5 × 105 cells/ml. 200 μl of cell suspension was seeded in a well of 96 well plates. The culture was incubated for 12 h to form cell monolayer. The original culture medium was then replaced with 100 μl maintenance medium containing 1% FBS. After 12 h, the cell monolayers were scratched vertically down the center of each well with a sterile 200-μl tip and washed three times with PBS, and then treated with 200 μl complete medium containing different concentrations of CMCS. Cells treated with complete medium were used as the control. Each assay was repeated three times and all experiments were performed in sextuplicate wells.

The migration distance in each well was recorded with the T1-SM 100 inverted microscope (Nikon Co. Japan) at time points of 0 h, 12 h and 24 h. Relative migration distance at each time point is calculated by subtracting the distance at time 12 h or 24 h from the distance at time 0 h. The effect of CMCS on the 2-dimensional migration of BEL-7402 cells was calculated using the percentage of inhibition rate (IR/%) with the following formula:

$$\mathrm{IR}(\%) = \left(1 - \frac{\text{relative migration distances in the CMCS treated groups}}{\text{relative migration distances in the control group}}\right) \times 100$$

2.5. In vitro 3-dimensional migration assay

Transwell membrance (6.5 mm diameter inserts, 8.0 μm pore size) was used in this assay. BEL-7402 cells (5×10^4 cells/100 μl) resuspended in RPMI 1640 medium containing 1% FBS were added to the cell culture inserts (8 μm pore size) and FBS (10% V/V) was used as chemoattractant in the bottom chamber. The cells in upper inserts was incubated with CMCS (5.0 mg/ml, 4.0 mg/ml, 3.0 mg/ml, 2.0 mg/ml and 1.0 mg/ml) and allowed to migrate for 24 h at 37 °C in a humidified incubator of 5% CO_2. The cells were fixed in methanol and stained with 0.5% crystal violet. Photographs of BEL-7402 cells migrated to the bottom surface of the insert in different groups were taken at 200 × magnification under the Eclipse E200 microscope (Nikon Co. Japan). For quantification purposes, 50% acetic acid was added to each well to dissolve crystal violet and the stain intensities were measured as absorbance under 570 nm using a Multiskan Go 151 Microplate Scanning Spectrophotometer (Thermo Fisher Scientific, INC, USA). Each assay was repeated three times and all experiments were performed in sextuplicate wells. The effect of CMCS in the 3-dimensional assay on the migration of BEL-7402 cells was calculated using the percentage of inhibition rate (IR/%) according to the formula below:

$$\mathrm{IR}(\%) = \left(1 - \frac{\text{absorbance of the CMCS treated groups}}{\text{absorbance of the control group}}\right) \times 100$$

2.6. Immunohistochemistry for MMP-9

Immunohistochemical staining was used to detect the expression of MMP-9. BEL-7402 cells were seeded in sterile glass slides (20 mm × 20 mm) in 6-well plates at a density of 8×10^4 cells/well in 2.0 ml of growth medium and incubated for 24 h. Five ml of complete medium containing CMCS was then added to each well to give the final concentrations of CMCS at 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml. The wells with complete medium only were used as controls. The culture was incubated for 4 to 5 days. For immunohistochemical staining, glass slides were first washed in 37 °C with pre-warmed phosphate buffered solution (PBS, pH 7.20) (3 × 1 min), then the BEL-7402 cells were fixed in 4% paraformaldehyde solution for 20 min. After three 3-min washes in PBS, the slides were incubated with 3% hydrogen peroxide in methanol for 15 min to quench endogenous peroxidase. The slides were incubated for 20-min with 5% bovine serum albuminin PBS to block nonspecific protein-binding sites. The slides were incubated at 4 °C overnight with a 150 dilution of the MMP-9 antibody followed by incubation with HRP-conjugated goat anti-rabbit IgG for 30 min at 37 °C. After 5–10 min staining with diaminobenzidine, the slides were counterstained with hematoxylin. Slides were observed and photographed using alight microscope. All photomicrographs were taken with a DT300 digital camera. Each assay was repeated three times and all experiments were performed in triple wells.

MMP-9 protein expression was quantified by Imagepro plus software and the percentage MMP-9 was calculated by adjusting the intensity option density (IOD) of control cells to 100. The effect of CMCS on the MMP-9 expression of BEL-7402 cells was calculated using the percentage of inhibition rate (IR/%) according to the formula below:

$$\mathrm{IR}(\%) = \left(1 - \frac{\text{IOD in CMCS treated groups}}{\text{IOD in control group}}\right) \times 100$$

2.7. Establishment of metastatic liver tumor model in mice

Cryopreserved H22 cells were cultured and passaged three times in mouse peritoneal cavity before being used for the metastatic liver tumor model. H22 cells from mouse ascites were