

INHIBITORY EFFECT OF CHITOSAN OLIGOSACCHARIDE ON HUMAN HEPATOMA CELLS *IN VITRO*

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Abstract

Background: Chitosan oligosaccharide, the degradation products of chitin, was reported to have a wide range of physiological functions and biological activities. In this study, we explored the inhibitory effect of Chitosan oligosaccharide on human hepatoma cells

Materials and Methods: MTT assay was applied to detect cell viability of the human hepatoma cells treated with Chitosan oligosaccharide. Flow cytometric analysis was used to investigate the apoptosis of the human hepatoma cells treated with Chitosan oligosaccharide. We employed western blot to investigate the underlying mechanisms involved in the apoptosis.

Results: Our data indicated that chitosan oligosaccharide dose-dependently inhibited the growth of hepatoma cells and induced apoptosis. On the molecular level, chitosan oligosaccharide decreased Bcl-2 and increased Caspase-3 expression which may be related to the apoptosis of hepatoma cells.

Conclusion: Our results provide an experimental basis for the clinical development of Chitosan oligosaccharide as a novel anti-hepatoma drug.

Key words: Chitosan oligochitosan, Hepatoma cells, Apoptosis, Bcl-2, Caspase-3.

Abbreviations: COS: Chitosan oligosaccharide, PI: Propidium iodide, ATCC: American Type Culture Collection, DMSO: Dimethyl sulphoxide, 5-Fu: 5-Fluorouracil, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, PBS: Phosphate buffered saline, SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis NC: Nitrocellulose membrane, RIPA: Radio Immunoprecipitation Assay, ECL: Esports Champion League SMMC-7721: Human hepatocellular carcinoma, ANOVA: Analysis of Variance.

Introduction

Hepatoma is a common malignancy and its incidence is recently rising in the developing world. More than 700,000 new cases are diagnosed throughout the world and more than 600,000 deaths are due to hepatoma each year (Renumathy et al., 2012). Conservative chemotherapy is not efficient for hepatoma patients because of drug resistance and toxic side effects (Meng et al., 2012). Therefore, it is urgent to find a novel therapeutic drug.

Chitosan oligosaccharide (COS), obtained by hydrolysis or degradation of chitin (Xu et al., 2007), has 3-10 saccharide (N-acetyl-glucosamine or glucosamine) residues (Han et al., 2005). Crustacean shells rich with Chitosan oligosaccharide have been used in Chinese medicine for long period. Studies indicated that chitosan oligosaccharide has a wide range of biological activities such as immunity regulation, anti-tumor, antioxidant and liver protection (Yin et al., 2009; Chakrabarti et al., 2004). However, the inhibition effect of chitosan oligosaccharide on hepatoma was rarely reported.

The aim of this study was to explore the inhibition effect of chitosan oligosaccharide and the underlying mechanisms on hepatoma cells. Our study will provide an experimental basis for the clinical development of novel anti-hepatoma drug.

Materials and Methods

Cell culture

SMMC-7721 cells were obtained from ATCC. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37 °C.

MTT assays

The cells (4×10^4 /well) were plated in 96-cell plates. After treated with COS (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/ml) for 24 h and 48 h, the viability of the cancer cells was detected by MTT assay. PBS (20 μ l) and 5-Fluorouracil (5-Fu) (0.5 mg/ml) were added as the negative control and the positive control, respectively. Each concentration of COS was repeated in six wells. The cells were cultured for 24 h and 48 h and incubated with 20 μ l MTT solution (5 mg/ml) for 4 h. The formazan crystals were dissolved in 150 μ l DMSO and the absorbance at 589 nm was determined with a Multiskan Ascent plate reader.

Hoechst 33258 fluorescent staining to detect apoptosis

The cells (1.5×10^5 /well) were seeded into 6-well plate. After 24 h COS (2.5 mg/ml) treatment, the cells were fixed in 1 ml of 4% (V/V) formaldehyde at 4 °C for 10 min. Then 1 ml staining solution of Hoechst 33258 was added into each well and was kept in dark for 10 min at room temperature. The cells were observed at the wavelength of 340 nm by a fluorescence microscopy.

Cell apoptosis by flow cytometry

The cells (1.5×10^5 /well) were seeded into 6-well plate. After 24 h COS (2.5 mg/ml) treatment, the cells were harvested and washed by PBS. Then the cells were stained with Annexin-V and PI solution for 10 min in dark and analyzed by flow cytometry.

Western blot analysis

The cells were harvested and solubilized in cold RIPA buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose (NC) membranes by semi-dry apparatus for 40 min and then blocked with blocking buffer for 1 h. The specific primary antibody 1:500 at optimized dilution was added and incubated overnight at 4 °C. After incubation with secondary antibody 1:3000 for 2 h, protein bands were visualized by ECL kit.

Statistical analysis

Data were expressed as the mean \pm SD. Data are expressed as the mean \pm SD. All statistical analyses were performed with standard statistical programs SPSS 11.0. A one-way ANOVA was used for the statistical analysis. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of COS on proliferation of SMMC-7721 cell

To evaluate the effect of COS on the growth of hepatoma, the MTT assay was performed. Our results showed that COS (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/ml) significantly inhibited the proliferation of SMMC-7721 cells (hepatoma cells) at low concentration groups ($P < 0.05$). Hepatoma cancer cells treated with COS showed a reduced proliferation capacity in a dose-dependent manner ($P < 0.001$). IC_{50} of COS on hepatoma cells for 24 h was 2.5 mg/ml (Figure 1).

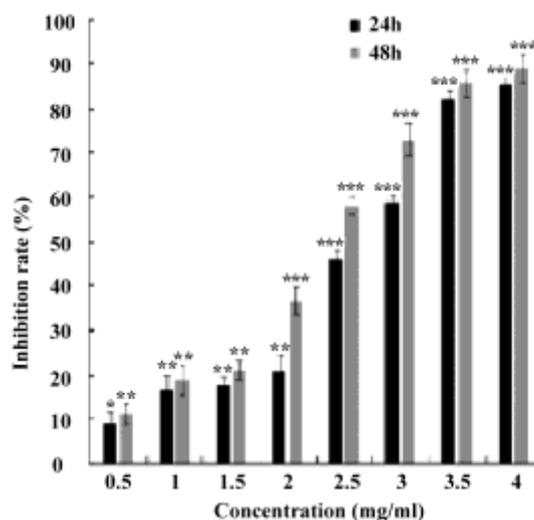


Figure 1: Effect of COS on the proliferation of hepatoma cancer cells. SMMC-7721 cells were treated without or with different concentration of COS for 24 h and 48 h. The inhibition rate of the cell proliferation was examined by MTT assay and expressed using the equation: inhibiting rate(%) = $(1 - \text{Abs test} / \text{Abs cont}) \times 100\%$. “*” compared with negative control group, $p < 0.05$. “**” compared with negative control group, $p < 0.01$. “***” compared with negative control group, $p < 0.001$.

Morphological changes observed by Hoechst 33258 fluorescent staining

After the fluorescent dye Hoechst 33258 staining, SMMC-7721 cells showed weak blue fluorescence and distributed regularly. However with the COS treatment (2.5 mg/ml), the fluorescence intensity of SMMC-7721 cells became much stronger than the negative control group which indicating apparent apoptosis (Figure 2).

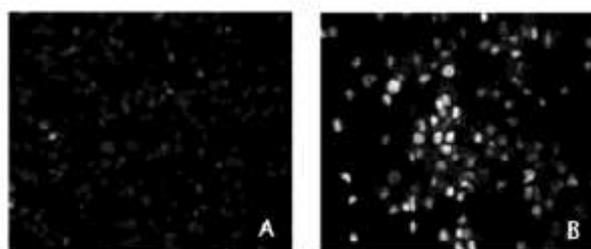


Figure 2: SMMC-7721 cells Hoechst 33258 Fluorescent staining apoptotic morphology. SMMC-7721 cells were treated without or with COS (2.5 mg/ml) for 24 h and observed under the microscope (40 x). (A) SMMC-7721 cells treated without COS. (B) SMMC-7721 cells treated with COS.

The effect of COS on the apoptosis of SMMC-7721 cells

The Annexin V/PI double staining results showed that SMMC-7721 cells of the negative control group were mainly distributed in the lower left quadrant (FITC-/PI-). However with COS treatment (2.5 mg/ml), the SMMC-7721 cells were mainly distributed in the upper right quadrant (FITC+/PI+) and the late apoptotic cells increased up to 76.04% (Figure 3). Our results indicated that the COS treatment could induce apoptosis on hepatoma cells.

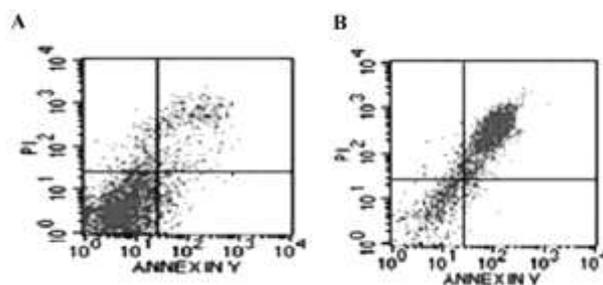


Figure 3: The effect of COS on the apoptosis of SMMC-7721. SMMC-7721 cells were treated without or with COS (2.5 mg/ml) for 24 h and detected by flow cytometry. (A) SMMC-7721 cells were treated without COS. (B) SMMC-7721 cells were treated with COS. The early and late apoptosis cells were indicated by the lower right and upper right quadrants.

The expression of Bcl-2 and Caspase-3 examined by Western blotting

The increase of Bcl-2 expression can prevent the release of cytochrome c and thus caspase activation (Pinton et al., 2000). Caspase-3 activation induces apoptosis via the cleavage of substrates known as mediators of apoptosis (Tanel et al., 2005). To further investigate the underlying molecular mechanism of COS on apoptosis, the expression of Caspase-3 and Bcl-2 were detected by Western blotting. Our western blotting results indicated that the expression of Cleaved-Caspase-3 and Bcl-2 were regulated in the COS treated SMMC-7721 cells (Figure 4).

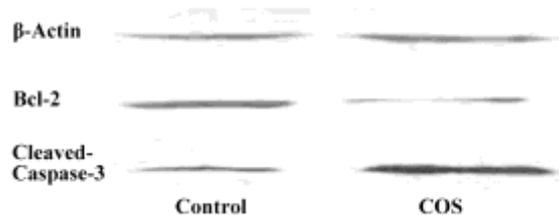


Figure 4. Effect of COS on the expression of Bcl-2 and Caspase-3. SMMC-7721 cells were treated with COS (2.5 mg/ml) for 24 h. The expression of Bcl-2 and Caspase-3 were detected by western-blotting. β -Actin served as loading control. The experiments were repeated three times.

Discussion

Hepatoma is the seventh most common cancer and the third leading cause of cancer-related deaths in the world (Yang et al., 2010). This study aimed to explore inhibition effect of COS on hepatoma cells. The results demonstrated that low concentrations of COS significantly inhibited the growth of hepatoma cells and the inhibition rate increased with the concentration of COS increasing. In addition, low concentrations of COS induced apoptosis on hepatoma cells was related to down-regulated expression of Bcl-2 and up-regulated expression of Caspase-3.

Our data indicated that the low concentrations of COS significantly inhibited the proliferation of SMMC-7721 cells ($P < 0.05$). IC₅₀ for SMMC-7721 cells at 24 h was 2.5 mg/ml. Previous study has showed that a 21-kDa water-soluble chitosan and oligochitosan can reduce the tumor growth in sarcoma 180-bearing mice which was similar with our results (Maeda et al., 2004).

We further investigated the underlying mechanisms of inhibiting effect of COS on the proliferation of hepatoma cells. The staining results and flow cytometry results both indicated that COS-treated cells induced apoptosis. The inhibition effects on the growth of hepatoma cells by COS might be related to apoptosis. The Bcl-2 protein was a potent

anti-apoptotic protein (Vaux et al., 1988; Berard et al., 2004) and Caspase-3 was a key enzyme apoptotic cascade in mammals (Corbiere et al., 2004; Nakamura et al., 2004; Tsujimoto et al., 2000). Insight into molecular mechanisms showed that Bcl-2 expression was decreased and Caspase-3 was increased in hepatoma cells following 24 h COS treatment. It is suggested that COS might induce apoptosis by up-regulating expression of Bcl-2 and down-regulating expression of Caspase-3.

Conclusion

In conclusion, our data is the first one to indicate that COS could inhibit the growth of hepatoma cells. Furthermore, COS can induce apoptosis which was related to the expression of down-regulated Bcl-2 and up-regulated Caspase-3. Therefore, we believed that the COS described in this study has potential to be developed as novel anti-cancer drug for hepatoma patients.

Acknowledgments

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