Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 71 (2008) 509–514
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Chitooligosaccharides induce apoptosis of human hepatocellular carcinoma cells via up-regulation of Bax

Qingsong Xu [a,c], Jiangli Dou [a,c], Peng Wei [a,c], Chengyu Tan [a], Xiaojing Yun [b], Yihong Wu [b], Xuefang Bai [a], Xiaojun Ma [a], Yuguang Du [a,*]

[a] *Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China*
[b] *Gene Research Center, Shanghai Medical College of Fudan University, Shanghai 200032, China*
[c] *Graduate School of Chinese Academy Sciences, Beijing 100049, China*

Received 13 May 2007; received in revised form 23 June 2007; accepted 27 June 2007
Available online 5 July 2007

Abstract

Chitooligosaccharides (COS) have been shown to regulate various cellular and biological functions. However, the effect of COS on apoptosis of hepatocellular carcinoma cells remains unclear. In this study, the activity and mechanism of COS against human hepatocellular carcinoma cells (SMMC-7721 cells) were investigated *in vitro*. The experiments showed that COS notably induced the apoptosis of SMMC-7721 cells and increased the cleavage of poly(ADP-ribose) polymerase. It presented a dose-dependent manner, and the apoptotic rate amounted to about 38% after treatment with 0.8 mg/ml COS for 72 h. The mRNA and protein levels of Bax were up-regulated by COS. These results demonstrated that COS induced apoptosis of SMMC-7721 cells. The possible mechanism is that COS up-regulate pro-apoptotic protein Bax, and trigger the cells a start-up of the apoptosis program.

Keywords: Chitooligosaccharides; Hepatoma; Apoptosis; Bax

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors (Farmer, Rosove, Shaked, & Busuttil, 1994) and it has the characteristics of long delitescence, rapid growth rate, strong malignance, easy invasion and metastasis, bad prognosis and so on. The treatment of inoperable HCC remains unsatisfactory. To improve the prognosis and the quality of life in those patients with inoperable HCC, it is necessary to search for effective drugs. As thus, induction of apoptosis in hepatoma cells with anticancer drugs would be probably a promising approach of liver cancer's therapy and it also may provide a new clew to the treatment of other cancers.

Chitosan, composed of β-(1 → 4)-linked *N*-acetyl-D-glucosamine (GlcNAc unit) and deacetylated glucosamine ($GlcNH_2$ unit) are obtained by deacetylation of chitin, a major component of exoskeleton in crustaceans and also a cell wall component of fungi. Chitooligosaccharides (COS) are partially hydrolyzed products of chitosan, which have various biological activities including antimicrobial activity (Choi et al., 2001; Tarsi, Corbin, Pruzzo, & Muzzarelli, 1998), antioxidant activity (Park, Je, & Kim, 2003; Xie, Xu, & Liu, 2001), immuno-enhancing effects (Feng, Zhao, & Yu, 2004), and antitumor activity (Tsukada et al., 1990). Those activities attracted more attentions, especially the activity of antitumor. The antitumor activity of COS was first reported in early 1970s (Muzzarelli, 1977). This activity was suggested mainly due to its cationic property exerted by amino groups, and later it was accepted that the molecular weight also plays a major role for the antitumor activity (Qin, Du, Xiao, Li, & Gao, 2002). Recently, it was proved that strong electronic charge is an important factor for anti-

* Corresponding author. Tel./fax: +86 411 84379060.
E-mail address: articles1805@gmail.com (Y. Du).

0144-8617/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2007.06.022

cancer activity of COS (Huang, Mendis, Rajapakse, & Kim, 2006). In addition, immunostimulation property of COS is also thought to be responsible for antitumor activity (Suzuki et al., 1986). Furthermore, some researchers found that antitumor effects of COS were due to increased activity of natural killer lymphocytes as observed in Sarcoma 180-Bearing Mice (Maeda & Kimura, 2004).

Though the antitumor activity of COS has been studied *in vivo* and *in vitro*, the molecular mechanisms of the antitumor are still unclear. In the present study, we investigated the activity of COS against the human hepatocellular carcinoma cells (SMMC-7721 cells) and apoptosis mechanism.

2. Materials and methods

2.1. General chemicals

Human hepatocellular carcinoma cells (SMMC-7721 cells) were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Chitooligosaccharides were prepared from enzymatic hydrolysis of chitosan (the degree of *N*-acetylation is below 5%) and separated with membrane according to the report (Zhang, Du, Yu, Mitsutomi, & Aiba, 1999). The TOF-MS profiles of COS showed the sample was composed mainly of chitooligosaccharides having degree of polymerization (DP) 3–9 (Zhao, She, Du, & Liang, 2007). Fetal calf serum, Dulbecco's modified Eagle medium (DMEM), and Trizol reagent were purchased from Gibco/BRL. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), β-tubulin antibody, propidium iodide (PI), and Hoechst 33258 were purchased from Sigma Chemical. Anti-poly (ADP-ribose) polymerase (PARP) and anti-Bax antibodies were purchased from Cell Signaling. Goat anti-rabbit and rabbit anti-mouse horseradish peroxidase (HRP) second antibodies and enhanced chemiluminescence (ECL) assay kit were purchased from Amersham.

2.2. SMMC-7721 cells culture

Human hepatocellular carcinoma cells (SMMC-7721 cells) were cultured in DMEM containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in 5% CO_2 incubator.

2.3. Analysis of viability of cells

Viability of cells was evaluated by monitoring the capacity of the cells to reduce the MTT. SMMC-7721 cells were cultured in 96-well plate in DMEM for 24 h, then replaced the medium with DMEM containing different concentrations of COS and cultured continuously. SMMC-7721 cells without treatment with COS would be used for control. Six replicate wells were used in each point in the experiments. The extent of MTT reduction was measured spectrophotometrically at 570 nm (Mosmann, 1983) by using a Bio-Rad 550 ELISA microplate reader. The inhibition rate of cell growth was calculated by the following formula: mean value of (control group-treated group)/control group × 100%.

2.4. Analysis of nuclear morphology by fluorescence staining

SMMC-7721 cells grown on the glass coverslips were treated with different dose of COS for 72 h and then the fluorescence staining (Hoechst 33258) was performed as previously described (Li et al., 2005).

2.5. Detection of apoptosis by flow cytometry

Apoptotic cells were detected by flow cytometry using propidium iodide. Briefly, after a treatment with different dose of COS for 72 h, adherent and non-adherent cells were collected and processed as previously described (Li et al., 2005). Stained cells were analyzed on a FACScan flow cytometer (Becton–Dickinson FACStar). The percentage of apoptosis was quantified from sub-G_1 events.

2.6. RNA extraction and RT-PCR analysis

RT-PCR was performed to determine changes in p53, Fas, Bcl-2, and Bax genes expression. After treatment with COS, total SMMC-7721 cells mRNA samples were prepared with Trizol reagent according to manufacturer's instructions. Samples (1.0 μg) were used as templates to perform the RT-PCR assay. The RT-PCR was performed according to the manual (TaKaRa). The cDNA was subjected to denaturation at 95 °C for 5 min, followed by 35 cycles (94 °C 45 s, 53 °C 45 s, and 72 °C 1 min) and incubated at 72 °C for 10 min and 4 °C for 5 min. The sequences of the primers used were as follow Table 1. PCR products were electrophoresed on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

2.7. Western blot analysis

SMMC-7721 cells were treated with different concentrations of COS for 72 h, and then protein of each group was collected. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto a PVDF membrane. The membrane was blocked for 2 h in a non-fat dried milk solution (5% in Tris-buffered saline) containing 0.5% Tween 20. The membrane was then incubated for 2 h at room temperature with primary antibody diluted 1:2000. Membrane-bound horseradish peroxidase-labeled protein bands were monitored with enhanced chemiluminescent reagents and chemiluminescent signals were detected using X-ray film.

2.8. Statistical analysis

The results were presented as means ± *SD*. Significant differences were evaluated with Student's *t* test.

Table 1
The sequences of the primers

Gene	Sense and antisense	PCR product (bp)	Reference
p53	5′-ctgaggttggctctgactgtaccaccatcc-3′ 5′-ctcattcagctctcggaacatctcgaagcg-3′	371	Wang et al. (2005)
Bcl-2	5′-ttgtggccttctttgagttcg-3′ 5′-tactgctttagtgaacctttt-3′	332	Agarwal et al. (1999)
Fas	5′-agactgcgtgccctgccaaga-3′ 5′-caggatttaaggttggagatt-3′	413	Agarwal et al. (1999)
Bax	5′-accagctctgagcagatcatg-3′ 5′-gggattgatcagacacgtaag-3′	626	Zhang et al. (2006)
β-Actin	5′-gggtcagaaggattcctatg-3′ 5′-ggtctcaaacatgatctggg-3′	237	Wang et al. (2005)

3. Results and discussion

3.1. COS led SMMC-7721 cells viability decline and morphology changes

The effect of COS on the cells viability was measured by the MTT assay, which reflects the cellular reducing activity. MTT assay as shown in Fig. 1 indicated that COS inhibited the SMMC-7721 cells proliferation in a concentration and time dependent manner. Morphological changes of cells were examined to investigate whether the observed cells death caused by COS related to apoptosis or not. SMMC-7721 cells cultured with different concentrations of COS for 72 h showed condensed nuclear morphology, increased fluorescence of the chromatin, and the presence of apoptotic bodies (as arrows shown in the Fig. 2). These results indicated that the cells were probably undergoing apoptosis.

3.2. COS induced apoptosis in SMMC-7721 cells

Apoptosis is a form of self-regulated cell death, which differs from necrosis. The characteristics of morphological changes of cell apoptosis include membrane blebbing, chromatin condensation, and the formation of apoptotic bodies. Apoptosis, essential in controlling cell number in many developmental and physiological settings, has been found to be impaired in many human tumors, suggesting that disruption of apoptotic function contributes substantially to the transformation of a normal cell into a tumor cell (Johnstone, Ruefli, & Lowe, 2002). To further confirm the apoptosis in SMMC-7721 cells was caused by COS, the DNA hypoploidy and cell cycle phase were analyzed by a flow cytometric method. In ethanol-fixed cells, the intensity of propidium iodide staining correlates with DNA content. Analysis of cell cycle distribution after SMMC-7721 cells cultured with COS for 72 h indicated that COS altered cell cycle progression. The apoptotic fraction visible as a sub-G_0/G_1 peak was 4.95% in the untreated cells. After the cells treated with COS at the concentration of 0.8 mg/ml, the apoptotic fraction increased to 37.87% (Fig. 3). Using flow cytometry, a typical apoptotic peak was observed. SMMC-7721 cells incubated with COS also were revealed to have morphological changes under fluorescent microscopy (Fig. 2). These results indicated that COS could stimulate apoptosis of SMMC-7721 cells. Some papers also described the antitumor potential and inducing apoptosis of COS using different cell lines. Pae et al. demonstrated that water-soluble chitosan oligomer induced HL60 cell differentiation to a granulocytic pathway in 4 days and apoptosis in 6 or 8 days (Pae et al., 2001). Huang et al. reported that chitooligosaccharides derivatives had anti-cancer activities in HeLa, Hep3B and SW480 three cancer cell lines (Huang et al., 2006). However the molecular mechanisms of the antitumor are still to be investigated.

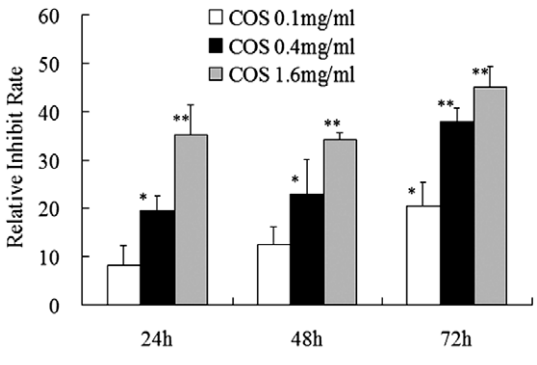

Fig. 1. SMMC-7721 cells proliferation was inhibited by COS. The inhibition rate of COS on SMMC-7721 cells proliferation was determined using the MTT method. SMMC-7721 cells were incubated with COS (0.1, 0.4, and 1.6 mg/ml) for 24, 48, and 72 h. Control cells were incubated in the absence of COS. The results were presented as mean ± *SD* of data from six independent experiments. Statistical significance $^{*}P < 0.05$ or $^{**}P < 0.01$ as compared to the control.

3.3. Effect of COS on p53, Fas, Bcl-2, and Bax mRNA expression

There are two pathways in apoptosis in mammalian cells. In the extrinsic death receptor pathway, caspase activation occurs as a direct consequence of death receptor ligation with upstream caspase-8 cleaving and activating effector caspases (caspase-9 and caspase-3) (Orrenius, Zhivotovsky, & Nicotera, 2003). In the intrinsic mitochondrial pathway, various death signals induce mitochondrial membrane potential (MMP) disruption, cytochrome *c*

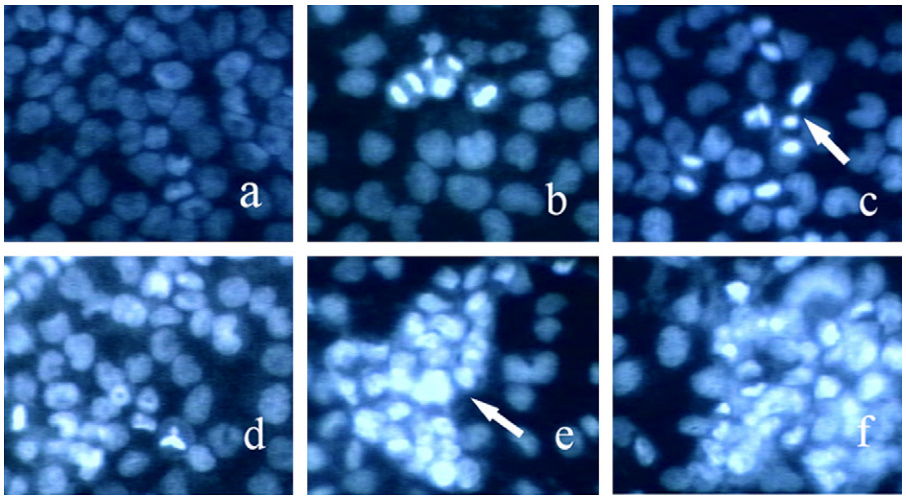

Fig. 2. Morphology changes of SMMC-7721 cells treated with COS. Hoechst 33258 staining of nuclei from SMMC-7721 cells was observed. SMMC-7721 cells were treated with COS at the concentrations of 0, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/ml for 72 h (above a–f). Images were visualized at 200× using a fluorescent microscope.

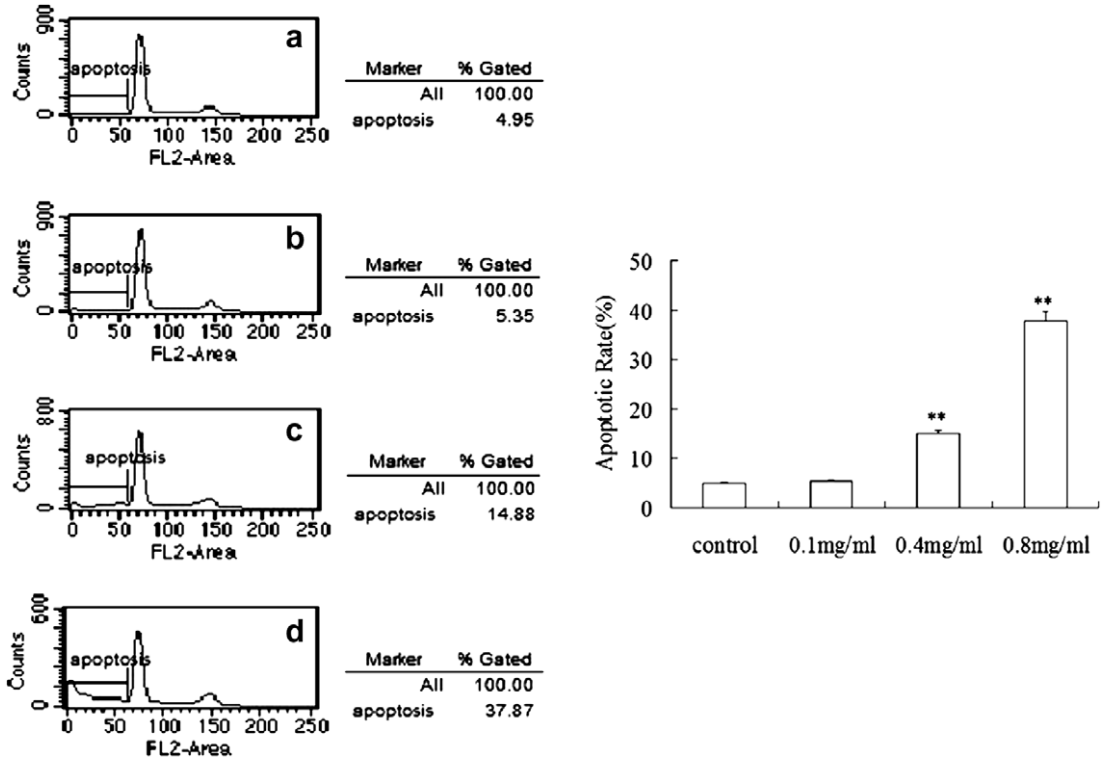

Fig. 3. Effect of COS on SMMC-7721 cells assayed by flow cytometry. The cells were incubated for 72 h with (a) medium alone, (b) 0.1 mg/ml COS, (c) 0.4 mg/ml COS, and (d) 0.8 mg/ml COS. Results were expressed as a percentage of the sub-G_1 population obtained from the cells of flow cytometry data of propidium iodide fluorescence.

release, and Apaf-1 activation followed by pro-caspase-9 and caspase-3 activation (Orrenius et al., 2003). To clarify the apoptosis pathway of SMMC-7721 cells induced by COS, we assayed the levels of p53, Fas, Bcl-2, and Bax mRNA, respectively. Total RNA was isolated after 72 h incubation. Fig. 4a showed that p53 and Fas mRNA levels remained slightly changed after COS treatment as compared with control. However, the Bax mRNA expression in the cells treated with COS was enhanced in a concentration-dependent manner, but the Bcl-2 mRNA change was unconspicuous (as shown in Fig. 4b). The functional activity of p53 most closely associated with tumor suppression is its role as a sequence-specific DNA binding protein and transcription factor that controls the expression of a large panel of gene products implicated in engagement of apoptosis (Dlamini, Mbita, & Zungu, 2004). Fas binding to its

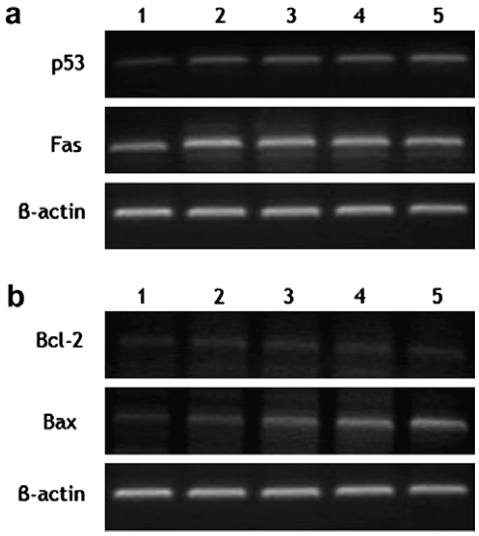

Fig. 4. Qualitative evaluate of p53, Fas, Bcl-2, and Bax mRNA in SMMC-7721 cells upon COS exposure. SMMC-7721 cells were treated with COS at the concentrations of 0, 0.1, 0.4, 0.8, and 1.6 mg/ml for 72 h (lanes 1–5). Total RNA was extracted and subjected to RT-PCR. The agarose gels were photographed and scanned. (a) p53 and Fas mRNA expression. (b) Bcl-2 and Bax mRNA expression.

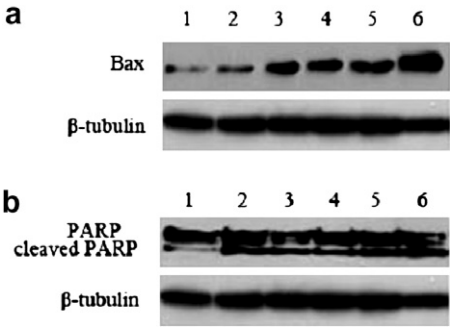

Fig. 5. Effect of COS on Bax expression and PARP cleavege. SMMC-7721 cells were treated with COS at the concentrations of 0, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/ml for 72 h (lanes 1–6). Then the cells were harvested and analyzed by western bolt. (a) Protein levels of Bax were analyzed with anti-Bax antibody. (b) PARP cleavages were analyzed with anti-PARP antibody. β-Tubulin served as a loading control.

ligand (Fasl) initiate the cell apoptosis. Fas receptor, which belongs to the tumor necrosis factor (TNF) receptor family, is activated by Fas ligand (Xiang, Fan, & Xu, 2003). Bcl-2 and Bax belong to Bcl-2 family. The over-expression of anti-apoptotic Bcl-2 has been shown to block the release of cytochrome *c* in response to a variety of apoptotic signals. On the contrary, the pro-apoptotic Bax promote cytochrome *c* release from the mitochondria (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999). These result suggested that COS induced apoptosis were mainly mediated by mitochondrial pathways.

3.4. COS up-regulated Bax in pro-apoptosis pathway

Bax is a pro-apoptotic Bcl-2 family protein (Oltvai, Milliman, & Korsmeyer, 1993) that resides in the cytosol and translocates to mitochondria upon induction of apoptosis. It has been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome *c* and caspases activation (Dlamini et al., 2004). Mitochondria are the target of the pro-apoptotic protein Bax (Er et al., 2006). From the Fig. 4b, we proved that COS could up-regulate the Bax mRNA expression, whether the Bax protein expression also could be regulated by COS? As the Fig. 5a shown, the expression of Bax protein was prominently up-regulated in the cells treated with different concentrations of COS. PARP is an 116 kDa nuclear poly(ADP-ribose) polymerase involved in DNA repair and cell proliferation. It can be cleaved into two parts (24 kDa and 89 kDa) by caspase-3, and serve as a marker of cells undergoing apoptosis. As shown in Fig. 5b, the 89 kDa fragment of PARP in cells treated with COS was much more compared to the control, and the increase was in line with the Bax increasing. Therefore, these results indicated that the COS induced apoptosis in SMMC-7721 via the up-regulation of Bax expression and activation caspases.

4. Conclusion

To our knowledge, we firstly reported that chitooligosaccharides could induce human hepatocellular carcinoma cells (SMMC-7721 cells) apoptosis in the present study and the possible mechanism is that COS up-regulate the Bax expression, trigger the cells a start-up of the apoptosis program. It may provide a new clew to the treatment of hepatoma.

Acknowledgements

We thank Prof. Jianxin Gu for instructing the experiments. This research was supported by "Knowledge Innovation project" of Chinese Academy of Sciences (No. KSCX 2-3-02-04).

References

Agarwal, R., Talati, M., Lambert, W., Clark, A. F., Wilson, S. E., Agarwal, N., et al. (1999). Fas-activated apoptosis and apoptosis mediators in human trabecular meshwork cells. *Experimental Eye Research, 68*, 583–590.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., & Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. *Annual Review of Cell and Developmental Biology, 15*, 269–290.

Choi, B. K., Kim, K. Y., Yoo, Y. J., Oh, S. J., Choi, J. H., & Kim, C. Y. (2001). In vitro antimicrobial activity of a chitooligosaccharides mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. *International Journal of Antimicrobial Agents, 18*, 553–557.

Dlamini, Z., Mbita, Z., & Zungu, M. (2004). Genealogy, expression, and molecular mechanisms in apoptosis. *Pharmacology and Therapeutics, 101*, 1–15.

Er, E., Oliver, L., Cartron, P. F., Juin, P., Manon, S., & Vallette, F. M. (2006). Mitochondria as the target of the pro-apoptotic protein Bax. *Biochimica et Biophysica Acta, 1757*, 1301–1311.

Farmer, D. G., Rosove, M. H., Shaked, A., & Busuttil, R. W. (1994). Current treatment modalities for hepatocellular carcinoma. *Annals of Surgery, 219*, 236–247.

Feng, J., Zhao, L., & Yu, Q. (2004). Receptor-mediated stimulatory effect of oligochitosan in macrophages. *Biochemical and Biophysical Research Communications, 317*, 414–420.

Huang, R., Mendis, E., Rajapakse, N., & Kim, S. K. (2006). Strong electronic charge as an important factor for anticancer activity of chitooligosaccharides (COS). *Life Sciences, 78*, 2399–2408.

Johnstone, R. W., Ruefli, A. A., & Lowe, S. W. (2002). Apoptosis: A link between cancer genetics and chemotherapy. *Cell, 108*, 153–164.

Li, Z., Wang, H., Zong, H., Sun, Q., Kong, X., Jiang, J., et al. (2005). Downregulation of beta 1,4-galactosyltransferase 1 inhibits its CDK11(p58)-mediated apoptosis induced by cycloheximide. *Biochemical and Biophysical Research Communications, 327*, 628–636.

Maeda, Y., & Kimura, Y. (2004). Antitumor effects of various low-molecular-weight chitosans are due to increased natural killer activity of intestinal intraepithelial lymphocytes in sarcoma 180-bearing mice. *Journal of Nutrition, 134*, 945–950.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55–63.

Muzzarelli, R. A. A. (1977). *Chitin*. London: Oxford Pergamon Press, pp. 262–270.

Oltvai, Z. N., Milliman, C. L., & Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell, 74*, 609–619.

Orrenius, S., Zhivotovsky, B., & Nicotera, P. (2003). Regulation of cell death: The calcium–apoptosis link. *Nature Reviews Molecular Cell Biology, 4*, 552–565.

Pae, H. O., Seo, W. G., Kim, N. Y., Oh, G. S., Kim, G. E., Kim, Y. H., et al. (2001). Induction of granulocytic differentiation in acute promyelocytic leukemia cells (HL-60) by water-soluble chitosan oligomer. *Leukemia Research, 25*, 339–346.

Park, P. J., Je, J. Y., & Kim, S. K. (2003). Free radical scavenging activity of chitooligosaccharides by electron spin resonance spectrometry. *Journal of Agricultural and Food Chemistry, 51*, 4624–4627.

Qin, C., Du, Y., Xiao, L., Li, Z., & Gao, X. (2002). Enzymic preparation of water-soluble chitosan and their antitumor activity. *International Journal of Biological Macromolecules, 31*, 111–117.

Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S., & Suzuki, M. (1986). Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose. *Carbohydrate Research, 151*, 403–408.

Tarsi, R., Corbin, B., Pruzzo, C., & Muzzarelli, R. A. (1998). Effect of low-molecular-weight chitosans on the adhesive properties of oral streptococci. *Oral Microbiology and Immunology, 13*, 217–224.

Tsukada, K., Matsumoto, T., Aizawa, K., Tokoro, A., Naruse, R., Suzuki, S., et al. (1990). Antimetastatic and growth-inhibitory effects of *N*-acetylchitohexaose in mice bearing Lewis lung carcinoma. *Japanese Journal of Cancer Research, 81*, 259–265.

Wang, A. G., Chu, Q. L., He, W. H., Xia, T., Liu, J. L., Zhang, M., et al. (2005). Effects on protein and mRNA expression levels of p53 induced by fluoride in human embryonic hepatocytes. *Toxicology Letters, 158*, 158–163.

Xiang, Q., Fan, M. Z., & Xu, B. (2003). Apoptotic induction of human lung carcinoma A549 cells by DFMO through Fas/FasL pathway. *Ai Zheng, 22*, 1260–1263.

Xie, W., Xu, P., & Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic & Medicinal Chemistry Letters, 11*, 1699–1701.

Zhang, H., Du, Y., Yu, X., Mitsutomi, M., & Aiba, S. (1999). Preparation of chitooligosaccharides from chitosan by a complex enzyme. *Carbohydrate Research, 320*, 257–260.

Zhang, M. S., Zhou, Y. F., Zhang, W. J., Zhang, X. L., Pan, Q., Ji, X. M., et al. (2006). Apoptosis induced by short hairpin RNA-mediated STAT6 gene silencing in human colon cancer cells. *Chinese Medical Journal, 119*, 801–808.

Zhao, X., She, X., Du, Y., & Liang, X. (2007). Induction of antiviral resistance and stimulary effect by oligochitosan in tobacco. *Pesticide Biochemistry and Physiology, 87*, 78–84.