

Chitosan Prevents the Development of AOM-Induced Aberrant Crypt Foci in Mice and Suppressed the Proliferation of AGS Cells by Inhibiting DNA Synthesis

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Abstract We study the effect of fungal-derived chitosan on the development of chemical-induced colonic precancerous lesions in ICR mice and delineate its possible molecular mechanisms. In the 2 weeks preventive experiments, mice fed with a diet containing high molecular weight chitosan (HMWC) had significant fewer aberrant crypt foci formation than those fed with control diet. As the treatment extended to 6 weeks, both low molecular weight chitosan (LMWC)- and HMWC-fed mice contained less aberrant crypt foci when compared to control. However, such effect was not observed in mice in the 6 weeks therapeutic experiments. The anti-tumorigenesis effect of water-soluble chitosan oligomer (WSCO) was tested on four cancer cell lines. WSCO significantly suppressed AGS and to a less extent, COLO 205 cells proliferation. Flow cytometry analysis of cell cycle distribution indicated that the percentage of S phase reduced significantly in AGS cells treated with WSCO together with a decrease in DNA synthesis rate in BrdU incorporation assay. WSCO treatment also upregulated cell cycle-related genes p21/Cip and p27/Kip, whereas downregulated that of PCNA. *J. Cell. Biochem.* 100: 1573–1580, 2007. © 2007 Wiley-Liss, Inc.

Key words: chitosan; anti-tumorigenicity; cells proliferation; cell cycle

INTRODUCTION

Most clinical tumors have a long history of pathological development during which they pass through several preneoplastic and prema-

lignant stages before becoming malignant. This situation offers the opportunity to interrupt or reverse tumor development at a still harmless stage, for instance by chemoprevention, that is, by taking medicines acting on distinct molecular processes of tumorigenesis [Marks and Furstemberger, 2000]. In recent year, considerable emphasis has been placed on identifying new cancer chemopreventive agents which could be useful for human populations [Levi et al., 2001]. Colon cancer is a major cause of cancer mortality and morbidity both in the USA and worldwide [Anonymous, 1997; Bailar and Gornik, 1997]. The aberrant crypt foci (ACF) of the colon stained with methylene blue in animals treated with colon-specific carcinogens have long been presumed to be preneoplastic

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lesions [Bird, 1987; Bird, 1995]. ACF assays have been widely used for detection of colorectal carcinogenesis modifiers within a short period in rodents initiated with the colon carcinogens 1,2-dimethylhydrazine or azoxymethane (AOM) [McLellan et al., 1991; Thorup et al., 1994; Tanaka et al., 1997].

Chitin, a polymer composed of β (1,4)-N-acetyl glucosamine units with a α -helical structure stabilized by intramolecular hydrogen bonding [Kas, 1997], is found in various nature products such as exoskeletons of insects, cell wall of some fungi, and microorganisms [Arroyo-Begovich et al., 1978; Lopez-Romero and Ruiz-Herrera, 1978]. Chitin can be partially hydrolyzed into chitosan which is a polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine with increased water solubility [Kafetzopoulos et al., 1993] and naturally present in some fungi and microorganisms [Singla and Chawla, 2001]. Therefore, the term chitosan is used to describe a series of chitosan polymers with different molecular weights, viscosity, and degree of deacetylation [Illum, 1998].

Chitosan has been reported to exert multiple biological effects including hypocholesterolemic [Sugano et al., 1988], anti-microbial [Chen et al., 1998], wound healing accelerating [Okamoto et al., 1995], gastric mucosa protecting [Acikgoz et al., 1995], anti-genotoxic [Ohe, 1996] and anti-cancer effects [Tokoro et al., 1988; Murata et al., 1991; Hasegawa et al., 2001]. One report has demonstrated that dietary chitosan supplement could reduce the development of chemical-induced precancerous lesions in the murine colon [Torzsas et al., 1996]. However, the molecular mechanisms underlying the anti-tumorigenesis effects of chitosan are still not fully understood. In this study, we used a well-established ACF model induced by AOM to test the anti-tumor effects of fungal-derived chitosan, and also explored the possible molecular anti-tumorigenesis mechanisms using gastrointestinal cell culture system.

MATERIALS AND METHODS

Experimental Animal

Male, 10- to 12-week, 30–35 g ICR mice (Laboratory Animal Center, National Cheng Kung University, or National Laboratory Animal Center, Taipei, Taiwan) were housed

in plastic cage (five mice/cage), with wire tops and wood-chip bedding and in a control room with 12/12 (light/dark) cycle with ventilation rate of 12 L/h, humidity of $60 \pm 5\%$ and temperature of $23 \pm 2^\circ\text{C}$. Food and water were available ad lib. All experimental animal care and treatment followed the guidelines set up by the Institutional Animal Care and Use Committee.

Chitosan and Azoxymethane

Chitosan was isolated from biomass of *Actinomyces taiwanensis* by modifying the methods described by Rane and Hoover [1993]. The degree of deacetylation of chitosan was determined by the first derivative UV-spectrophotometry method with a HITACHI U-2001 spectrophotometer [Tan et al., 1998]. The molecular weight of chitosan was measured by the method of SEC³ (Triple Detection GPC) in both the size exclusion chromatography mode and the triple detection mode combining refractive index (RI) detector with differential viscometer (DV) detector and light scattering (LS) detector. The buffer stock of chitosan sample in GPC mobile phase buffer (0.35 N acetic acid with sodium acetate 8.2 g/L, pH 4.1) was prepared at 2.5 mg/ml and was used for analytical method. The buffer stock was heated to 90°C for 3 h. The mobile phase for SEC³ was 0.35 N acetic acid with sodium acetate 8.2 g/L, pH 4.1 and the flow-rate was 0.7 ml/min. Sample injection volume was 100 μl and SEC³ separation was performed on a TSK GPC column G3000 PW_{XL} from Tosoh, Japan. Detectors used for triple detector analysis were the RI detector (WATERS 410), DV detector (Viscotek model T60), and LS detector (Viscotek model T60). All data were collected and processed with TriSEC software using the three detectors simultaneously. The degree of deacetylation in HMWC and LMWC preparations was 83.1% and 87.3%. HMWC and LMWC samples had molecular weights of 327.9 and 227.9 KDa, respectively. WSCO which have greater than three but less than ten saccharide residues was kindly provided by Mind-Ace Company (Miyazaki). Groups of mice were given azoxymethane (AOM, Sigma-Aldrich, St. Louis, MO) at a dose of 5 mg/kg via an i.p. injection twice a week for 2 weeks. Mice were given LMWC or HMWC supplement for 2 weeks along with (preventive experiment) or 2 weeks after AOM administration (therapeutic experiment). Body weights

were then monitored for 2 or 6 weeks for the preventive experiments or 8 weeks for the therapeutic experiments. For preparation chitosan supplement diet, a standard lab chow (Lab Diet 5010, Purina Mills, Inc.) was mixed with 2% (w/w) of LMWC, HMWC, or cellulose.

Determination of Aberrant Crypt Foci (ACF)

Mice were killed by an i.p. injection of overdose sodium pentobarbital (Nembutal). The entire colons were excised, cut longitudinally, rinsed with phosphate-buffered saline (PBS) and fixed flat between sheets of filter paper with 3.7% neutral formalin for overnight. The fixed colons were then placed in Ringer's solution containing 0.2% methylene blue for 20–30 min. After washing with PBS, the stained colons were placed luminal side up on a glass slide and kept in moist with Ringer's solution. ACF characterized as large, dark stained, elevated lesions were counted using a light microscope at a magnification of 100× in rectal (2 cm), middle (2 cm), and cecal (1 cm) areas [Bird et al., 1989]. Samples were examined blindly by two observers.

Cell Lines and Cell Culture

COLO 205 (human colon adenocarcinoma, CCL-222), AGS (human gastric cancer), T-24 (human bladder cancer), and Jurkat cells (human leukemia) (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (for COLO 205 and Jurkat cells), McCoy's 5A (for T-24), or 1:1 DME/F-12 (v/v) (for AGS) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml), and 0.3 mg/ml glutamine in a humidified incubator (37°C, 5% CO₂). WSCO was added at the indicated doses. For control specimens, same volume of H₂O was added instead of WSCO. Cell viability was estimated by trypan blue exclusion assay as previously described [Ho et al., 1997].

Cell Synchronization, WSCO Treatment, and Flow Cytometry Analysis

At 24 h after plating of cells, cells were washed three times with PBS and then incubated with medium containing 0.04% FCS for additional 24 h. Under such conditions, cells were arrested in G₀/G₁ as determined by flow cytometry analysis [Ho et al., 2005]. After serum starvation, the low-serum (0.04% FCS)

medium was removed and the cells were then challenged by addition of medium containing 10% FCS. WSCO (1,000 µg/ml) was added at various time points. The cell cycle stages in the WSCO and control groups were measured by flow cytometry analysis. Cells were harvested and stained with propidium iodide (50 µg/ml) (Sigma-Aldrich Chemical Co., St. Louis, MO), and DNA content was measured using a FACScan laser flow cytometer analysis system (Becton–Dickinson, San Jose, CA); and 15,000 events were analyzed for each sample.

BrdU Cell Cycle Analysis

Cells were plated in 10 cm dishes. After serum starvation with 0.04% FCS for 24 h to render them quiescent and to synchronize their cell cycle activities, the cells were returned to media with 10% FCS with 1,000 µg/ml of WSCO or control. AGS cells were harvested at various time points. BrdU (10 µg/10ml) was added 1 h before cell collection. Cells were then washed once with 1× PBS, fixed with 75% alcohol, stored in 4°C for 1 h, centrifuged at 500g, 10°C for 10 min, then pellets were collected. After denature DNA with 1 ml of 2N HCl/Triton X-100, pellets were collected after centrifugation at 500g for 10 min. Cells then were neutralized with 1 ml 0.1 M Na₂B₄O₇ · 10H₂O (pH 8.5). After centrifugation at 500g for 10 min, pellets then were treated with 1 ml 0.5% Tween-20/1%BSA/PBS to adjust cell concentration. After adding of 20 µl of anti-BrdU to cells (1 × 10⁶) at room temperature for 30 min, washed once with 1 ml of Tween-20/1%BSA/PBS, the pellets were collected by centrifugation at 500g for 10 min. Flow cytometry analysis was performed after adding propidium iodide.

Western Analysis

Western blotting analysis was performed as described previously [Lin et al., 2002]. Briefly, cell lysates were prepared, electrotransferred, immunoblotted with antibodies, and then visualized by incubating with the colorogenic substrates (nitroblue tetrazolium, NBT and 5-bromo-4-chloro-3-indolyl phosphate, BCIP) (Sigma Chemical Co., St. Louis, MO). The expression of GAPDH was used as the control for equal protein loading.

Statistics

Results were expressed as mean ± SE for each study. Data were analyzed by Student's *t*-test or

linear regression method. A *P*-value of 0.05 or less was considered statistically significant.

RESULTS

Co-Treatment but not Post-Treatment of LMWC and HMWC Reduces AOM-Induced ACF Formation in ICR Mice

We first tested whether LMWC or HMWC could have anti-tumorigenesis effect on the AOM-induced ACF formation in ICR mice. Co-treatment of HMWC but not LMWC with AOM for 2 weeks significantly suppressed ACF formation in mice as compared to cellulose control (Table Ia, *P* < 0.05). If the treatment extended to 6 weeks both LMWC and HMWC significantly suppressed ACF formation in mice as compared to cellulose control (Table Ib, *P* < 0.05). However, such effect was not noted in the 6-week therapeutic experiment (Table Ic). These results suggested that chitosan might interfere with the AOM-induced precancerous lesions formation in mice. ACF formation concentrated mostly in rectal area and least in cecal area in all three groups of experiments (Table I, right panel). Chitosan supplement did not affect the growth of mice. There were no significant differences of body weight among treatment and control groups (data not shown).

WSCO Suppresses Proliferation of Cancer Cells by Arresting Cell Cycle

To further study the anti-tumorigenesis effect of chitosan, we tested the anti-proliferative effect of WSCO on various cancer cell lines by

trypan blue exclusion assay. As shown in Figure 1a,b, WSCO at doses of 100 and 1,000 µg/ml significantly inhibited proliferation of AGS and colon cancer cells, but not T24 and Jurkat cells after 72 h of treatment (Fig. 1c,d). Since WSCO suppressed the growth of AGS cells more significantly than that of COLO 205 cells, we chose AGS cells for the following experiments. Further experiment indicated that WSCO suppressed the S phase of AGS cells started from 24 h and sustained for 48 h (Fig. 2).

Effect of WSCO on the BrdU Incorporation of AGS Cells

To further confirm that WSCO could suppress S phase cells of AGS, Brd U cell cycle analysis was conducted. As shown in Figure 3, treatment of synchronized AGS cells with WSCO significantly inhibited BrdU incorporation as compared to control with greatest difference occurred at 48 h after treatment (control vs. WSCO, 24.2% vs. 16.8%, respectively). This result indicated that WSCO-induced anti-proliferative effect of AGS cells might be through inhibition of S phase DNA synthesis.

Effect of WSCO on Cell Cycle Regulatory Genes of AGS Cells

Since the effect of WSCO on AGS cells was primarily through inhibition of S phase DNA synthesis, we next examined how WSCO regulated the expression of cell cycle associated genes by Western blotting analysis. Figure 4 shows that treatment of synchronized AGS cells

TABLE I. Effect of LMWC/HMWC on the Aberrant Crypt Formation and Distribution in ICR Mice

Group	No. of aberrant Crypt foci per colon	Distribution of aberrant crypt Foci in the colon		
		Rectal	Middle	Cecal
(A) 2 weeks preventive experiment				
Cellulose (n = 10)	6.2 ± 3.7	5.0 ± 3.2	0.9 ± 1.1	0.3 ± 0.5
LMWC (n = 10)	6.8 ± 4.4	5.3 ± 4.1	1.3 ± 1.2	0.2 ± 0.4
HMWC (n = 10)	3.8 ± 1.7*	2.9 ± 1.4	0.6 ± 0.8	0.3 ± 0.5
(B) 6 weeks preventive experiment				
Cellulose (n = 8)	8.1 ± 7.6	6.8 ± 7.1	1.1 ± 1.3	0.2 ± 0.4
LMWC (n = 8)	2.9 ± 2.2*	2.5 ± 2.0	0.5 ± 0.8	0.0 ± 0.0
HMWC (n = 8)	5.1 ± 4.5*	4.5 ± 4.2	0.6 ± 0.7	0.0 ± 0.0
(C) 6 weeks therapeutic experiment				
Cellulose (n = 10)	6.0 ± 5.2	3.9 ± 3.3	1.8 ± 1.7	0.3 ± 0.5
LMWC (n = 10)	5.7 ± 1.5	4.1 ± 1.1	1.5 ± 1.2	0.1 ± 0.3
HMWC (n = 10)	5.4 ± 4.5	3.9 ± 3.5	1.5 ± 1.5	0.1 ± 0.3

+, Values represent means ± SE.

**P* < 0.05, compare to cellulose control.

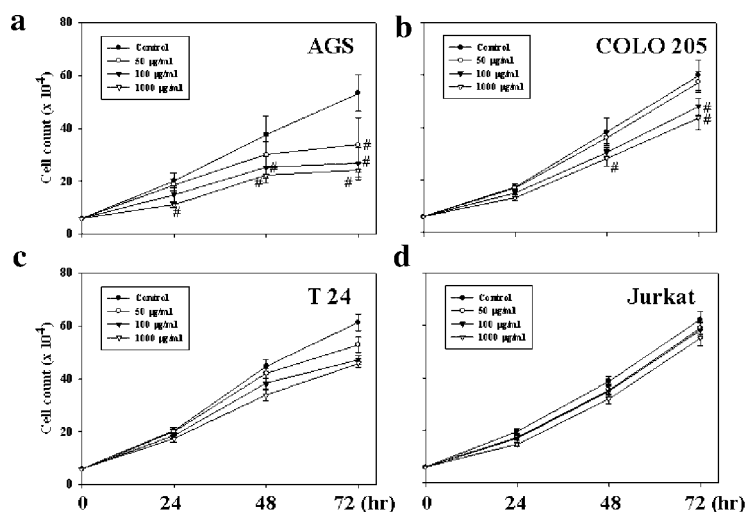


Fig. 1. Effect of WSCO on the proliferation of cancer cells. Human gastric cancer (AGS), human colon cancer (COLO 205), human bladder cancer (T24), and human leukemia (Jurkat) cells were treated with increasing concentrations of WSCO (0-1,000 $\mu\text{g/ml}$) for 1-3 days. Medium with or without WSCO were changed everyday until cell counting. Viable cells were determined by trypan blue exclusion assay. Data are the means \pm SE of one representative experiment performed in triplicates. # $P < 0.05$ versus controls.

with 1,000 $\mu\text{g/ml}$ of WSCO significantly decreased the expression of PCNA, and increased that of p21 and p27 with no effect on cyclins A, B, E, cdc-2, p19, CDK2 and CDK4 over time. These findings suggested that downregulation of PCNA and upregulation of p21 and p27 might be involved in the WSCO-induced suppression of S phase DNA synthesis in AGS cells.

DISCUSSION

In this study, we demonstrated that fungal-derived chitosan suppressed colon precancerous lesion development *in vivo*, and that suppression of DNA synthesis by modulation of cell cycle regulatory molecules might be the underlying molecular mechanisms involved in the chitosan-mediated inhibition of cancer cell growth.

One report has demonstrated high- and low-molecular weight animal-derived chitosan inhibited colon cell proliferation and ACF formation [Torzsas et al., 1996], in which CF1 mice were first pretreated with AOM for 2 weeks followed by a 6-week diet chitosan supplementation. In contrast to the previous report, our results showed that 2- and 6-week co-treatment of LMWC or HMWC with AOM (preventive experiments) significantly suppressed aberrant crypt formation in mice while no effect was observed in the 2-week AOM pretreatment

group (therapeutic experiment). The discrepancies might be explained as follows. First, male ICR instead of female CF1 mice were used in our study. Second, origin of chitosan was different. In our study, chitosan was obtained from fungi. Third, different control diets were used. In our study, 2% cellulose diet rather than AIN-76 standard diet were used in the control group. Bulk forming effect in suppression of aberrant crypt formation might be blunted by using appropriate fiber control diet. Insoluble fibers with their bulk-forming capacity and resistance to digestion by intestine microflora, have been shown to inhibit colon carcinogenesis [Harris et al., 1993]. The mechanisms might be dilution of bile acids or other toxic compounds in the colon and decreased contact between carcinogens in the stool and the colon mucosa [Gallaher et al., 1992].

Epidemiologic studies have demonstrated that consumption of diet high in plant food could lower the incidence of colon cancer formation [Burkitt, 1971; Greenwald et al., 1987; Kim, 2000]. Although animal studies have clearly shown the preventive effects of dietary fiber on the development of colon cancer, the underlying mechanisms are still not fully understood. In our study, WSCO suppressed colon and gastric cancer cell proliferation. Results of flow cytometry analysis and BrdU incorporation assay indicated that S phase DNA

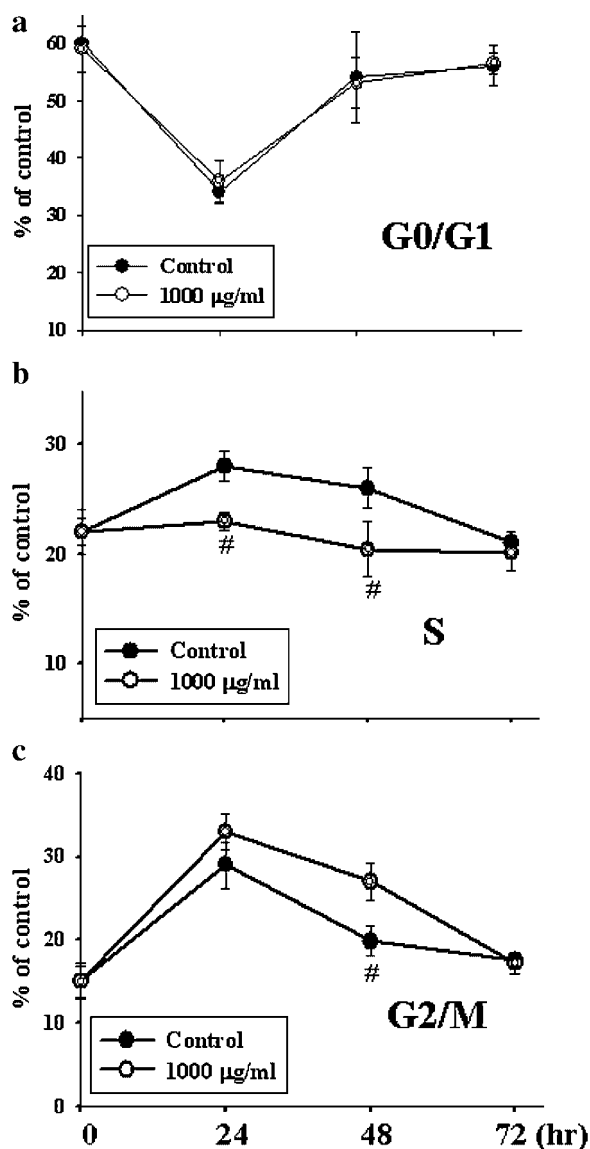


Fig. 2. Effect of WSCO on the cell cycle regulation of AGS cells. AGS cells were synchronized as described in the Materials and Methods, and were incubated in 10% FBS with or without 1000 µg/ml of WSCO for 1–3 days. DNA contents were analyzed by FACS and the percentage of cells in G0/G1, S, and G2/M phases of the cell cycle were determined using established CellFIT DNA analysis software. **a:** Effect of WSCO on G0/G1 phase distribution of AGS cells. **b:** Effect of WSCO on S phase distribution of AGS cells. **c:** Effect of WSCO on G2/M phase distribution of AGS cells. Data are the means \pm SE of one representative experiment performed in triplicates. # $P < 0.05$ versus controls.

synthesis was inhibited in gastric cancer cells treated with WSCO. Relatively little information is known for S-phase control in the cell cycle regulation mechanisms. It has been demonstrated that CDK inhibitors such as p21, p27, and p16 could be candidates for S-phase regulation [Ogryzko et al., 1997]. PCNA, a key

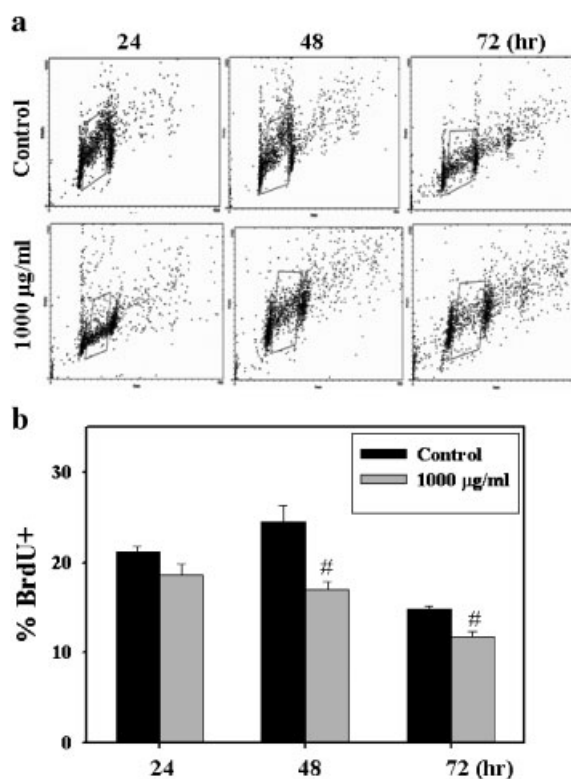


Fig. 3. Effect of WSCO on the BrdU incorporation of AGS cells. **a:** AGS cells were synchronized as described in the Materials and Methods section, and were incubated in 10% FBS with or without 1,000 µg/ml of WSCO for 1–3 days. BrdU incorporation was determined by FACS analysis. **b:** Percentage of cells with BrdU incorporation was quantitative. Data are the means \pm SE of one representative experiment performed in triplicates. # $P < 0.05$ versus controls.

molecule involved in DNA replication machinery, has also been associated with S-phase regulation [Dahm and Hubscher, 2002; Naderi et al., 2005]. Western blot data clearly showed that WSCO treatment to AGS cells resulted in increased expression of p21 and p27 as well as decreased expression of PCNA suggested that p21, p27, and PCNA were the key molecules involved in the chitosan-induced suppression of DNA synthesis in cancer cells. These findings also suggested that chitosan could directly regulate cell growth which might be the mechanism involved in our preventive experiments in mice.

Previous study has demonstrated that LMWC exerted anti-tumor activity in a sarcoma 180-bearing mice. The effect might in part be due to enhancement of nature killer activity in intestinal intraepithelial lymphocyte [Maeda and Kimura, 2004]. Induction of apoptosis of one bladder cancer cell line by chitosan was also reported [Hasegawa et al., 2001]. These results

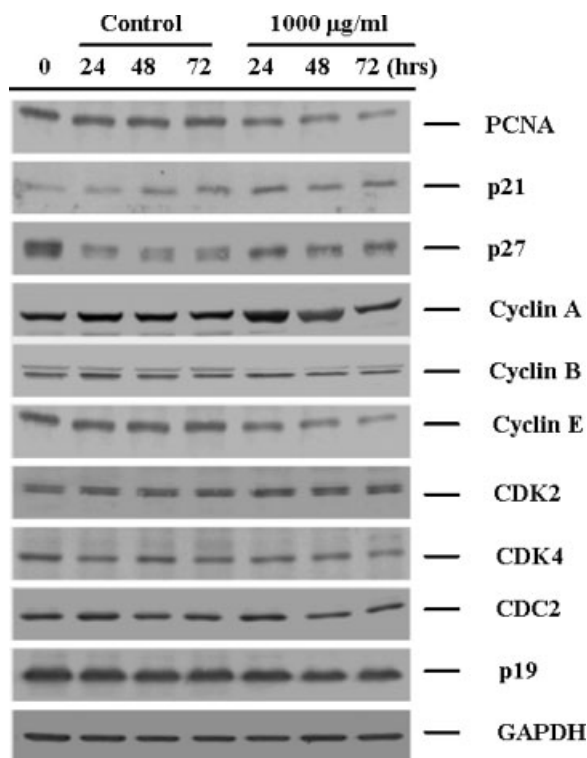


Fig. 4. Time-dependent effect of WSCO on the expression of S phase regulatory proteins in AGS cells. AGS were released from quiescence by incubation in culture medium supplement with 10% FBS containing 1,000 µg/ml of WSCO or control for 1–3 days. The cells were harvested and protein extracts were separated by SDS–PAGE. After electrophoresis, proteins were transferred into Immobilon-P membranes, probed with proper dilution of specific antibodies, and then detected by using the NBT/BCIP system. Membrane was also probed with anti-GADPH antibody to correct for difference in protein loading.

were consistent with our findings that chitosan exerted anti-tumor activity both in vitro and in vivo. According to our findings, we further proposed that inhibition of DNA synthesis in cancer cells by cell cycle regulatory proteins might be one of the possible underlying mechanisms.

In conclusion, chitosan can specifically inhibit colon precancerous lesion formation in mice and suppress gastric and colon cancer cell proliferation. The results from the present in vitro and in vivo studies highlight the molecular mechanism of chitosan-induced anti-tumorigenesis effect which might have potential application for the disease.

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