

## Inhibition of Proinflammatory Cytokine-induced Invasiveness of HT-29 Cells by Chitosan Oligosaccharide

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**Abstract** The effect of chitosan oligosaccharide (COS, 1 kDa <MW<3 kDa) on proinflammatory cytokines-induced nitric oxide (NO) production and invasiveness of human colorectal adenocarcinoma HT-29 cells was investigated. COS (0.1–5 mg/ml) suppressed the NO production induced by proinflammatory cytokines (100 U/ml IFN- $\gamma$ , 10 ng/ml IL-1 $\alpha$ , and 25 ng/ml TNF- $\alpha$ ) in HT-29 cells. Inducible nitric oxide synthase (iNOS) expression induced by these cytokines was inhibited by COS. COS pretreatment inhibited the invasiveness of cytokines-treated HT-29 cells through Matrigel-coated membrane in a dose-dependent manner. COS also inhibited cytokines-induced matrix metalloproteinase (MMP)-2 activity. This study shows that proinflammatory cytokines induce NO production, iNOS expression, and invasiveness of human colorectal adenocarcinoma HT-29 cells. COS pretreatment inhibited cytokines-mediated NO production, iNOS expression, and invasiveness of HT-29 cells. These results provide sufficient information for the further development of COS as an antitumor metastatic agent for the treatment of colon cancer.

**Keywords:** Chitosan oligosaccharide, invasion, matrix metalloproteinase-2, nitric oxide, proinflammatory cytokines

Increased nitric oxide (NO) production has been implicated in many of the pathophysiological processes that lead to the development and progression of colon cancer [18, 19]. Inducible nitric oxide synthase (iNOS) can be expressed in response to inflammatory stimuli [11] and is responsible for most NO production [12]. Overexpression of iNOS in colorectal cancer has been shown to be correlated with metastatic potential [1, 10].

Metastasis is a major complication of cancer disease. The invasion and metastatic progression of cancer cells require cell adhesion, proteolytic degradation of extracellular matrix,

and motility factors [2]. Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that have key roles in the growth and invasion of colorectal cancer [13]. The types of MMPs found in colorectal cancer are gelatinases and matrilysin [3]. Activation and overexpression of MMPs have been detected in colon carcinoma [15].

Chitin and chitosan have various biological activities including immunopotentiating, antitumor, antihypertensive, and antimicrobial actions [9, 14, 16, 17]. However, their high molecular weight and high viscosity may limit their uses *in vivo*. Therefore, chemical, fermentation, and enzymatic methods have been developed to prepare chitosan oligosaccharide (COS) [20]. The best yield of oligosaccharide with a high degree of polymerization was achieved by enzymatic hydrolysis [8].

In the present study, we investigated whether the NO induced by proinflammatory cytokines can modulate tumor cell invasiveness of human colorectal adenocarcinoma HT-29 cells, and the effect of COS on cytokine-induced NO production and invasiveness of HT-29 cells.

## MATERIALS AND METHODS

### Preparation of Chitosan Oligosaccharide

Water-soluble COS (1 kDa<MW<3 kDa) was prepared from 1% (w/v) chitosan in a dual reactor system [4]. Chitosan (1%, w/v) was dissolved in 0.27 M lactic acid, and the pH of the solution was adjusted to 5.5 with NaHCO<sub>3</sub>. The dual reactor system was composed of a column reactor packed with immobilized chitosanase (derived from *Bacillus pumilus* BN-262) and an ultrafiltration membrane reactor (Millipore Minitan system, molecular weight cut-off 3,000, and 1,000 membrane). Chitosan was partially hydrolyzed using the packed column reactor, and this partially hydrolyzed chitosan was then applied to a substrate feed tank with an ultrafiltration membrane reactor to produce COS.

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### Induction of Inducible Nitric Oxide Synthase

Confluent cell monolayers were treated with cytokines (100 U/ml IFN- $\gamma$ , 10 ng/ml IL-1 $\alpha$ , and 25 ng/ml TNF- $\alpha$ ) or cytokines plus COS (0.1–5 mg/ml) or 1400W (0.5 mM) in serum-free medium for 48 h.

### Measurement of Nitrite

Nitrite as an indicator of NO production was determined using Griess reagent [6] by measuring the absorbance at 570 nm. The protein concentration of supernatant was determined using a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO, U.S.A.) with bovine serum albumin as the standard.

### Western Blotting

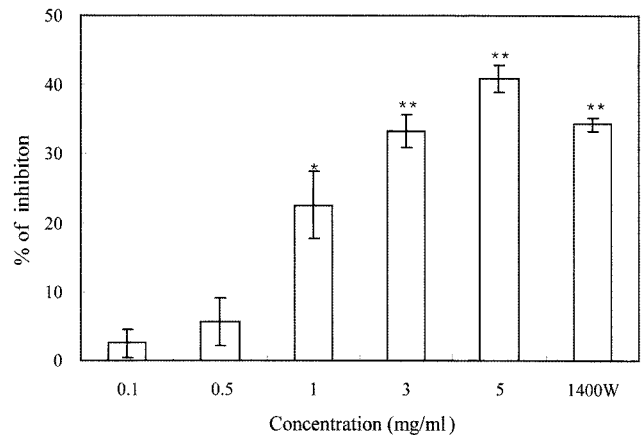
The protein from cell lysates (30  $\mu$ g/lane) was electrophoresed on SDS-polyacrylamide gel (7%), and then transferred onto a polyvinylidene difluoride membrane. The membrane was treated with 5% nonfat milk for 1 h and probed with primary antibodies to iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a final dilution of 1:1,000. Primary antibodies were detected using biotin-rabbit anti-mouse IgGAM (H+L) (Zymed, San Francisco, CA, U.S.A.) and alkaline phosphate-conjugated streptavidin, and visualized by 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate (Promega, Madison, WI, U.S.A.).

### Invasion Assay

Cell migration was measured using Transwell chambers (Corning Inc., Corning, NY, U.S.A.) with 8  $\mu$ m pore polycarbonate filters coated with matrigel matrix (BD Biosciences, Bedford, MA, U.S.A.). HT-29 cells ( $0.5 \times 10^4$  cells/well) were inoculated into the chamber and then incubated for 24 h to achieve cell adhesion. The cells were treated with cytokines plus COS or 1400W for 48 h, and the invading cells on the lower side were fixed with 100% methanol and stained with haematoxylin and eosin Y solution. The number of cells was counted under a microscope at 100 $\times$  magnification.

### Gelatin Zymographic Assay

HT-29 cells were treated with the mixture of cytokines plus COS or 1400W for 12 h. The culture medium was subjected to electrophoresis in a 10% SDS-PAGE gel containing 0.1% (w/v) gelatin. The gel was washed with 2.5% (v/v) Triton X-100 for 1 h, and then incubated at 37 $^{\circ}$ C for 24 h in 50 mM Tris-HCl (pH 7.5), 5 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, and 1  $\mu$ M ZnCl<sub>2</sub>. Thereafter, the gel was stained with 0.05% Coomassie blue in 45% (v/v) methanol/1% (v/v) acetic acid and destained in 10% acetic acid (v/v)/25% methanol (v/v). Unstained areas corresponded to zones of MMPs proteolytic activities.



**Fig. 1.** Effect of 48 h treatment with chitosan oligosaccharide (COS) on cytokines (100 U/ml IFN- $\gamma$ , 10 ng/ml IL-1 $\alpha$ , and 25 ng/ml TNF- $\alpha$ )-induced nitrite production by HT-29 cells. 1400W, 0.5 mM N-(3-[aminomethyl]benzyl)acetamide. Data shown are mean values with bars indicating the SD of the mean ( $n=3$ ). \* $p<0.05$ , \*\* $p<0.01$  compared with control.

### Statistical Analysis

The data were analyzed for statistical significance using the Student's *t*-test. *p* Values less than 0.05 were considered to be significant.

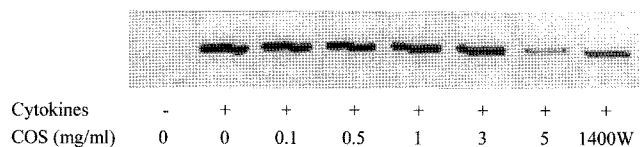
## RESULTS AND DISCUSSION

### Nitrite Production

Treatment of HT-29 cells with cytokines (100 U/ml IFN- $\gamma$ , 10 ng/ml IL-1 $\alpha$ , and 25 ng/ml TNF- $\alpha$ ) increased nitrite production. Chitosan oligosaccharide at 1–5 mg/ml significantly inhibited cytokine-induced nitrite production (1 mg/ml,  $p<0.05$ ; 3 and 5 mg/ml,  $p<0.01$ ) (Fig. 1). NO can facilitate metastasis by inducing angiogenesis and inhibiting platelet aggregation [5]. Excess production of nitric oxide has been implicated in the pathogenesis of colorectal cancer [19]. COS may therefore inhibit tumor progression and the process of metastasis.

### Expression of Inducible Nitric Oxide Synthase

iNOS expression in HT-29 cells was not detectable in the absence of cytokines. However, after the addition of cytokines, the expression of iNOS increased. Treatment of the cells with 5 mg/ml COS markedly reduced the expression of iNOS (Fig. 2). 1400W also inhibited cytokine-induced iNOS expression. Enhanced expression of iNOS in human colon carcinoma has been correlated with tumor growth and vascular invasion and could be indicative of survival potential [10, 21]. Thus, reduction of the iNOS expression may be able to facilitate the anti-inflammatory action and inhibit metastasis.



**Fig. 2.** Inhibitory effect of chitosan oligosaccharide (COS) on cytokines (100 U/ml IFN- $\gamma$ , 10 ng/ml IL-1 $\alpha$ , and 25 ng/ml TNF- $\alpha$ )-induced inducible nitric oxide synthase (iNOS) protein expression. 1400W, 0.5 mM N-(3-[aminomethyl]benzyl)acetamide.

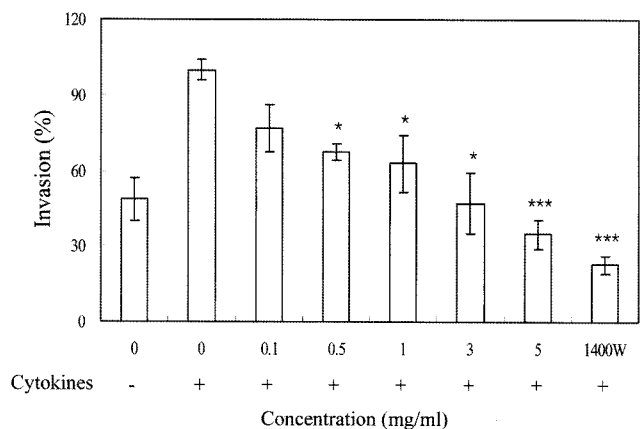
### Invasiveness of HT-29 Cells

COS inhibited the invasiveness of cytokine-treated HT-29 cells through Matrigel-coated membrane in a dose-dependent manner (Fig. 3). Cells invasiveness was inhibited by treatment with the iNOS inhibitor 1400W, demonstrating the contribution of iNOS in the process of tumor cell invasion. 1400W inhibited the NO production (Fig. 1) and expression of iNOS (Fig. 2) in HT-29 cells. Inhibition of NO production by 1400W was accompanied by a reduction of the Matrigel invasion of the HT-29 cells (Fig. 3). These results suggest that COS may exert an anti-invasive action by the same mechanism by which NO production and expression of iNOS was inhibited in HT-29 cells.

### Matrix Metalloproteinase (MMP)-2 Activity

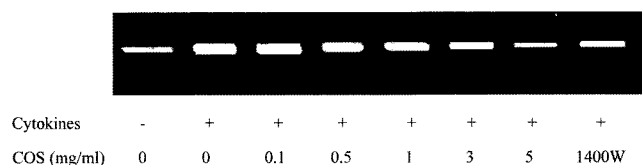
The inhibitory effect of COS on MMP-2 activity in HT-29 cells was dose-dependent (Fig. 4). MMPs are key enzymes involved in tumor cell metastasis and invasion through the proteolysis of several extracellular matrix proteins [7]. Thus, decreases in MMP-2 levels are associated with reduction in invasion.

In this present study, COS pretreatment inhibited cytokine-mediated NO production, iNOS expression, and



**Fig. 3.** Effect of chitosan oligosaccharide (COS) on cytokines (100 U/ml IFN- $\gamma$ , 10 ng/ml IL-1 $\alpha$ , and 25 ng/ml TNF- $\alpha$ )-induced invasiveness of HT-29 cells.

1400W, 0.5 mM N-(3-[aminomethyl]benzyl)acetamide. Data shown are mean values with bars indicating the SD of the mean (n=3). \* $p$ <0.05, \*\*\* $p$ <0.001 compared with the control.



**Fig. 4.** Effect of chitosan oligosaccharide (COS) on cytokines (100 U/ml IFN- $\gamma$ , 10 ng/ml IL-1 $\alpha$ , and 25 ng/ml TNF- $\alpha$ )-induced matrix metalloproteinase (MMP)-2 activity in HT-29 cells. 1400W, 0.5 mM N-(3-[aminomethyl]benzyl)acetamide.

invasiveness of HT-29 cells. These results suggest that COS may provide a promising source for the development of an antitumor metastatic agent for the treatment of colon cancer.

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