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Aminoethylated chitooligomers and their apoptotic activity on AGS human cancer cells

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ABSTRACT

In this study, the ability of aminoethylation of chitooligomers (COS) to inhibit the proliferation of AGS human gastric adenocarcinoma cells was evaluated using COS with lower molecular weight (<1 kDa). As water-soluble aminoderivatized COS derivatives, aminoethyl-COS (AE-COS), dimethyl aminoethyl-COS (DMAE-COS) and diethyl aminoethyl-COS (DEAE-COS) were synthesized and confirmed by their IR spectra results in comparison to previous study. Aminoderivatized COS-induced cell death was characterized by cell viability, changes in nuclear morphology and cell morphology. All aminoderivatized COS significantly inhibited cell proliferation of AGS cancer cells in a dose-dependent manner. Moreover, protein and gene expression levels of the regulators involved in apoptosis pathway such as Caspase-9, Bax, p53 and p21 were examined using RT-PCR and Western blot analysis. The exposure of synthesized COS derivatives to AGS cells induced apoptotic activity. Therefore, the present results suggest that all aminoderivatized COS derivatives have a promising potential as valuable as cancer chemopreventive agents.

1. Introduction

Chitosan, a natural nontoxic heteropolysaccharide composed of β-1,4-D-glucosamine and N-acetyl-D-glucosamine has been widely applied in the biomedical and pharmaceutical industries, due to its anti-microbial activity (Jeon, Park, & Kim, 2001; Kong, Kim, Ahn, Byun, & Kim, 2010), anti-viral activity (Artan, Karadeniz, Karagozlu, Kim, & Kim, 2010), antioxidant effect (Park, Je, & Kim, 2004) and anti-diabetic value (Karadeniz, Artan, Kim, & Kim, 2008) and anti-cancer significance (Kim & Rajapakse, 2005). Recent studies on chitosan have shown that there is interest in converting it into soluble chitooligomers (COS) obtained by either chemical depolymerization (Defaye & Guillot, 1994; Horowitz, Roseman, & Blumenthal, 1957; Tsukada & Inoue, 1981) or enzymatic hydrolysis of chitosan (Izume & Ohtakara, 1987; Muzzarelli, 2010; Muzzarelli, Stanic, & Ramos, 1999; Muzzarelli, Terbojevuch, Muzarelli, & Francescangeli, 2002; Muzzarelli, Tomasetti, & Ilari, 1994). The lowest oligomers of chitosan are not only water-soluble but are also even more versatile than chitosan (Qin, Du, Xiao, Li, & Gao, 2002).

Apoptosis is the principal form of programmed cell death, contributes to the development and the proper functioning of multicellular organisms through the removal of virus-infected cells and cancer cells (MacFarlane, 2003). Although most of the current anti-cancer therapies rely on the eradication of tumor cells, drugs promoting apoptosis may be effective against many cancers and should become an important strategy to counteract cancer (Fesik, 2005).

Gastric cancer is one of the most frequent causes of death: although the incidence and mortality have been decreasing, it is still the second most frequent cause of death around the world, after lung cancer (Lee, Yang, & Ahn, 2002). Sub-types of gastric cancer exist and this classification depends on the tissues where they originate: adenocarcinoma is the most common gastric cancer, being reported as cause of death in 95% of diagnosed subjects. The AGS cell line is a human gastric adenocarcinoma, widely used as a model system for evaluating cancer cell apoptosis (Liu et al., 2003).

In a previous study, water-soluble aminoderivatized COS with medium molecular weight were synthesized with different substituent groups and, new biological activities were evaluated (Karagozlu, Kim, Karadeniz, Kong, & Kim, 2010). Scope of the present study is the enhancement of the ability of aminoethylated COS to inhibit adenocarcinoma cell proliferation and to induce apoptosis.

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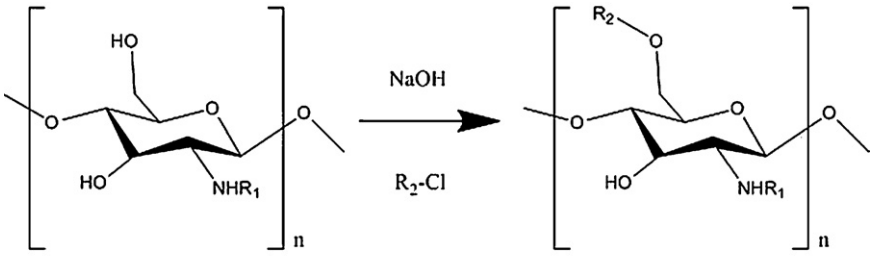

AE-COS: R_1= H or $COCH_3$; R_2= $(CH_2)_2NH_2$

DMAE-COS: R_1= H or $COCH_3$; R_2= $(CH_2)_2N(CH_3)_2$

DEAE-COS: R_1= H or $COCH_3$; R_2= $(CH_2)_2N(CH_2CH_3)_2$

Fig. 1. Synthesis and chemical structure of aminoethyl-COS derivatives (AE-COS, DMAE-COS and DEAE-COS).

2. Materials and methods

2.1. Synthesis of samples

Aminoethyl COS derivatives (Fig. 1) were synthesized by previous method (Je & Kim, 2006). 2-Aminoethyl chloride hydrochloride (Sigma–Aldrich Corp., St. Louis, MO, USA) for AE-COS, 2-(dimethylamino)ethyl chloride hydrochloride (Sigma–Aldrich Corp., St. Louis, MO, USA) for DMAE-COS and 2-(diethylamino)ethyl chloride hydrochloride for DEAE-COS (Sigma–Aldrich Corp., St. Louis, MO, USA) were used to prepare for their COS derivatives. COS (0.40 g) were mixed with 20 ml of 3.0 M amino hydrochloride and stirred at 50 °C. After 2 h, 20 ml of 3.0 M NaOH was added to the reaction mixture dropwise, and continuously stirred for 48 h. The reaction mixture was acidified with 0.1 M HCl and dialyzed against water. After 2 days, the product was freeze dried and COS derivatives were collected as fluffy brown powder. Synthesized COS was characterized by infrared spectrometer (Shimadzu IR-408, Tokyo, Japan) TLC and ^{1}H NMR (Jeol JNM-ECP-400, Tokyo, Japan) analysis.

2.2. Cell culture

Human gastric adenocarcinoma cell line AGS was cultured as monolayer in T-75 tissue flasks (Nunc, Roskilde, Denmark) in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA), 2 mM L-glutamine and 100 μg/ml penicillin–streptomycin. The cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C and subcultured by detaching with trypsin–EDTA solution 1–2 times each week at about 70–80% confluent.

2.3. Cell viability assay

To determine cell survive after exposure to different concentration of COS derivatives, cell proliferation was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as reported in our previous studies with a slight modification (Kong et al., 2010). Briefly, cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After incubation for 24 h, cells were treated with 0, 50 and 500 μg/ml concentrations of different samples and MTT was determined by measuring the absorbance at 540 nm using a microplate reader (GENios microplate reader, Tecan Austria GmbH, Salzburg, Austria). The percentage of untreated cell's viability was taken as control to calculate relative cell viability.

2.4. Morphological analysis and Hoechst staining

AGS cells treated by various concentrations of different samples were washed twice and fixed in 4% paraformaldehyde (Sigma–Aldrich Corp., St. Louis, MO, USA) in PBS (phosphate buffered saline) for 10 min at room temperature. The fixed cells were washed with PBS and their morphological changes were detected by a light microscope (CTR 6000; Leica, Wetzlar, Germany). Furthermore, nuclear deformation was detected by Hoechst 33342 staining. For Hoechst 33342 assay, AGS cells were treated by various concentrations of the samples for 12 h. Then, cells were washed twice and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were stained with 1 μg/ml of the fluorescent DNA-binding dye, Bisbenzimide Hoechst 33342 (Sigma–Aldrich Corp., St. Louis, MO, USA) and incubated for 1 h at room temperature to reveal nuclear condensation/aggregation. The Hoechst-stained cells were visualized and photographed under fluorescence microscope (CTR 6000; Leica, Wetzlar, Germany).

2.5. Flow cytometric assay

Fluorescence associated cell sorting assay was performed to determine apoptotic activity of the samples on AGS cells. The cells (5×10^5 cells/ml) were grown in culture flask for 24 h. Then various concentrations of COS samples were treated. After 24 h, cells were collected and washed with cold PBS. Cells (1×10^6 cells/ml) were re-suspended with binding buffer (BD Diagnostic Systems, Cockeysville, MD, USA) and 100 μl of the mixture transferred to 5 ml culture tube. Five microliters of both FITC Annexin V and PI (BD Diagnostic Systems, Cockeysville, MD, USA) were added to the tubes. Mixtures were gently vortexed and incubated for 15 min at room temperature in dark. Finally, 400 μl of binding buffer was added to the tubes and mixtures were analyzed by flow cytometer (BD Diagnostic Systems, Cockeysville, MD, USA) within 1 h.

2.6. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using a Trizol reagent (Invitrogen Co., Foster City, CA, USA) as reported in manufacturer's manual. Two micrograms of total RNA was reverse transcribed using M-MLV reverse transcriptase to give cDNA and a PCR reaction was performed using specific primers as reported previously (Karagozlu et al., 2010). The amplified PCR products were run in 1% agarose gels and visualized by ethidium bromide (EtBr) and visualized by UV

light using AlphaEase gel image analysis software (Alpha Innotech, San Leandro, CA, USA).

2.7. Protein isolation and Western blot analysis

AGS cells were grown at a density of 3×10^4 cells in cultured, treated with various concentrations of the samples for 24 h and protein of each group was collected. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) then transferred onto polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., Amersham, England) and blocked in 5% (w/v) skim milk. 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 visualized by chemiluminescence assay kit (Amersham Pharmacia Biotech., Amersham, England) using LAS-3000 image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.8. Statistical analysis

The data were presented as mean ± SD ($n = 3$). Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at $p < 0.05$. The statistical software package, SAS v9.1 (SAS Institute Inc., Cary, NC, USA), was used for these analyses.

3. Results and discussion

3.1. Synthesis of aminoethylated COS derivatives

The structures of aminoderivatized COSs such as AE-COS, DMAE-COS and DEAE-COS were confirmed from the IR spectra results with comparison with previous study (Je & Kim, 2006; Karagozlu et al., 2010). In the FT-IR spectrum of synthesized derivatives, the peaks of absorptions at 2965 cm^{-1} due to C–H stretching and 1000–1150 cm^{-1} due to C–O–C stretching were observed, supporting the occurrence of substitution. According to comparison, it was estimated that the C-6 hydroxyl group of COS was replaced by the substitution group of AE, DMAE and DEAE for AE-COS, DMAE-COS and DEAE-COS, respectively (data not shown). Moreover, The structures of aminoderivatized COSs such as AE-COS, DMAECOS and DEAE-COS were confirmed from the ^{1}H NMR spectrum results (Fig. 2) with comparison with previous study (Je & Kim, 2006). ^{1}H NMR spectrum of our samples showed a peak 2.9 ppm for methyl and characteristic ($-CH_2CH_2N-$) groups of substituted AE, DMAE and DEAE, although the substituted group's peak was not observed in ^{1}H NMR spectrum of COS.

3.2. Cell viability

In order to compare the effects of COS and aminoderivatized COS derivatives (AE-COS, DMAE-COS and DEAE-COS) on cell proliferation, the cells were exposed to increasing concentrations of COS and aminoderivatized COS derivatives for 24 h and cell viability was examined by MTT viability assay (Fig. 3). Exposure of AGS cells to increasing concentrations of aminoderivatized COS resulted in a dose-dependent decrease in cell viability relative to control cells. Treatment with COS for 24 h inhibited the proliferation with rates of approximately 8% and 23% at concentrations of 50 and 500 μg/ml, respectively. AE-COS inhibited the cell proliferation with rates of approximately 22% and 84% at concentrations of 50 and 500 μg/ml, respectively. DMAE-COS inhibited cell proliferation with rates of approximately 45% and 85% at concentrations of 50 and 500 μg/ml, respectively. Finally, DEAE-COS inhibited the cell proliferation with

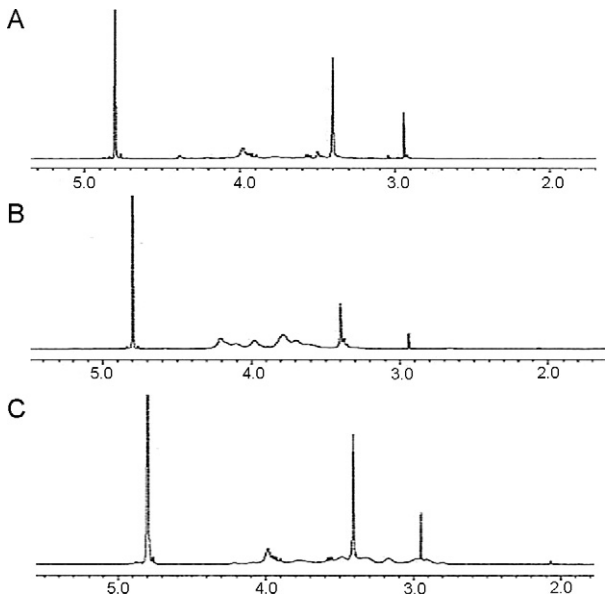

Fig. 2. ^{1}H NMR analysis of aminoethyl-COS derivatives (AE-COS (A), DMAE-COS (B) and DEAE-COS (C)) prepared from COS with low molecular weight.

rates of approximately 68% and 86%, respectively, under same exposure conditions. According to results of MTT assay, when 500 μg/ml of sample was treated, inhibitory effects on the proliferation of AGS cells for all three samples are quietly same. However, when the treated dose of the sample decreased, DEAE-COS shows maximum activity while AE-COS shows minimum activity. On the other hand, results of cell viability assay showed that aminoethylation of COS (below 1 kDa) significantly enhanced the anti-proliferative effect of COS (below 1 kDa) on AGS human adenocarcinoma cells.

3.3. Morphological changes and Hoechst 33342 staining

Morphological changes and the cell death of AGS cells were characterized using light microscope. The morphological changes after 24 h exposure to various concentrations of COS, AE-COS, DMAE-COS and DEAE-COS are shown in Fig. 4a. According to the results, the number of attached cells was remarkably reduced with increasing doses of aminoderivatized COS. Especially under high concentration of aminoethylated COS exposure, we observed that

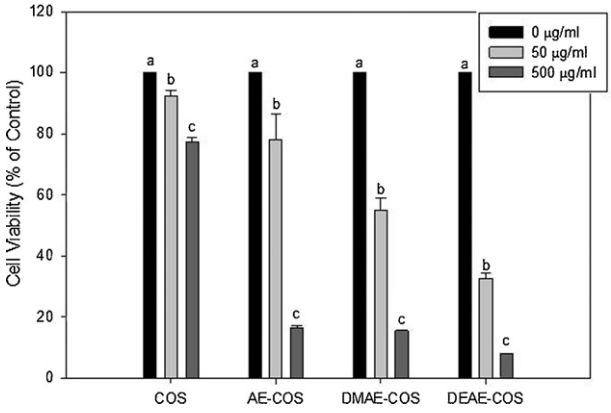

Fig. 3. Antiproliferative effects of COS and aminoderivatized COS derivatives in AGS cell line. Cells were treated with different concentrations of COS and aminoderivatized COS derivatives for 24 h. $^{a-d}$Symbolize that the different letters in the each sample are significantly different ($p < 0.05$) by Duncan's multiple range test.

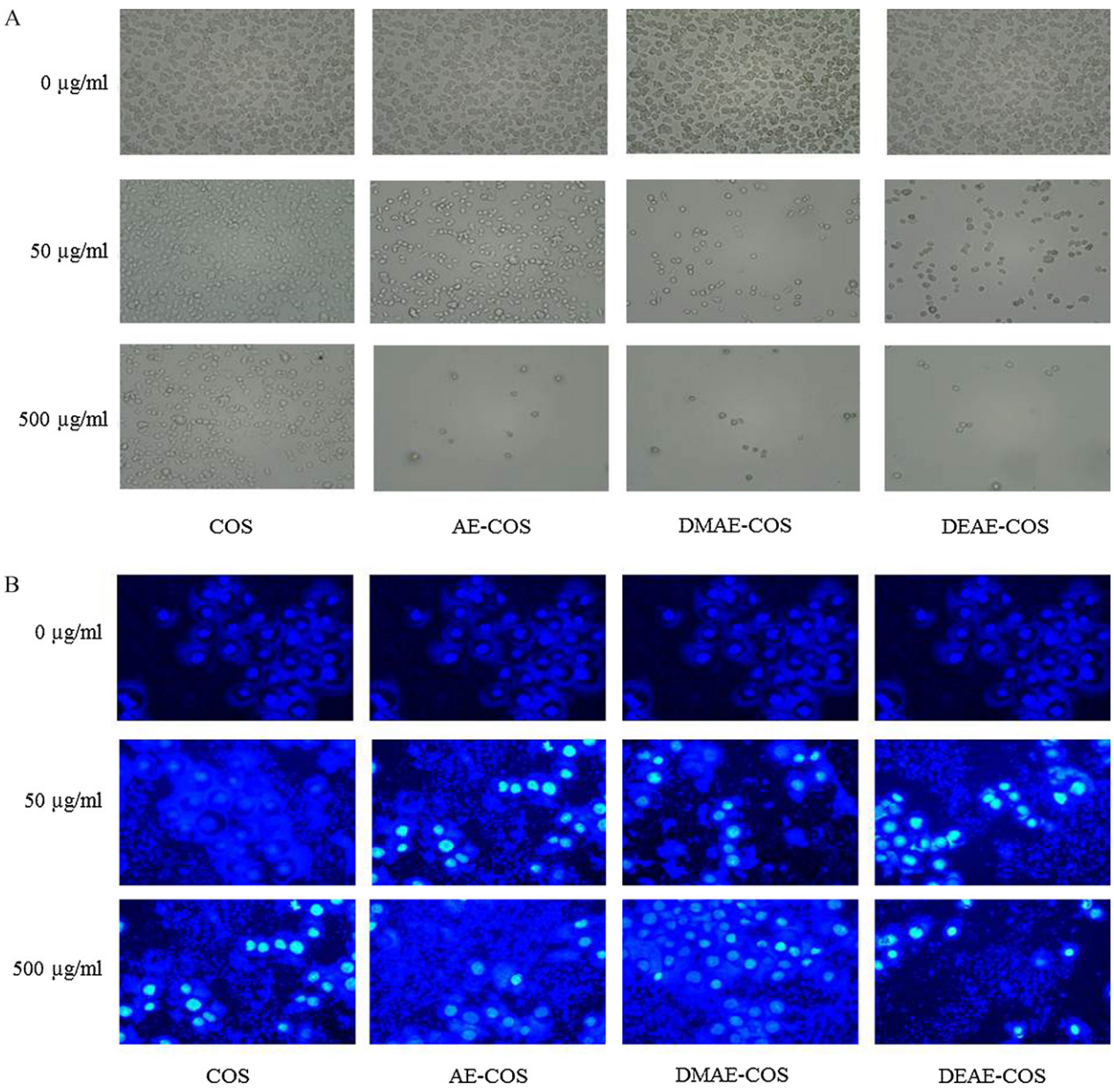

Fig. 4. Morphological changes in AGS cells by treatment with COS and aminoethyl-COS derivatives (AE-COS, DMAE-COS and DEAE-COS). Cells were grown and treated with COS and aminoethyl COS derivatives for 12 h. The medium was removed and attached cells were fixed with 4% paraformaldehyde and washed with PBS. Cell morphology was detected by light microscope (viewed at magnification of 100×) (A) and stained with Hoechst 33342 dye in PBS for 1 h in the dark and nuclear condition was detected by fluorescence microscope (viewed at magnification of 400×) (B).

most of the cell was detached. Also, morphological changes in attached cells were observed. Treatment with aminoethylated COS caused shrinking on cell shape. The comparison analysis exhibited that aminoethylation of COS enhanced the anti-proliferative effect on AGS cells. Furthermore, in order to determine whether the inhibitory effect of aminoderivatized COS on cell proliferation was due to the apoptotic cell death, AGS cells were stained with Hoechst 33342 dye after sample treatment for 12 h and the morphological changes of nuclear were observed under fluorescence microscope (Fig. 4b). The nuclear degradation of AGS cells can be observed under fluorescence lighting. Hence, the nuclei with chromatin concentration and apoptotic bodies were observed in the cells exposed to COS-derivatives with increasing of concentrations. This observation exhibits that aminoderivatized COS induce cell death in AGS cells through a typical apoptotic pathway.

3.4. Fluorescence activated cell sorting

An Annexin V binding assay was conducted to evaluate the apoptotic response in AGS cells. After 24 h treatment of samples, AGS cells stained for Annexin V binding assay and analyzed by FACS (Fig. 5). According to results, increased Annexin V staining was seen in AGS cells in the presence of aminoethylated COS for 24 h. The percentage of living cells which are at late apoptotic phase increased in a dose-dependent manner. For COS treated AGS cells, living cell ratio was 87.7%, 73.4% and 67.2% at concentrations of 0, 50 and 500 μg/ml, respectively. On the other hand, the results of living AGS cell ratio were 87.7%, 74.3% and 24.3%, respectively, in AE-COS treated cells. The ratios were 87.7%, 81.3% and 19.2%, respectively, in DMAE-COS treated cells, and were 87.4%, 31.3% and 12.7%, respectively, in DEAE-COS treated cells. However, the induction of apoptosis was higher

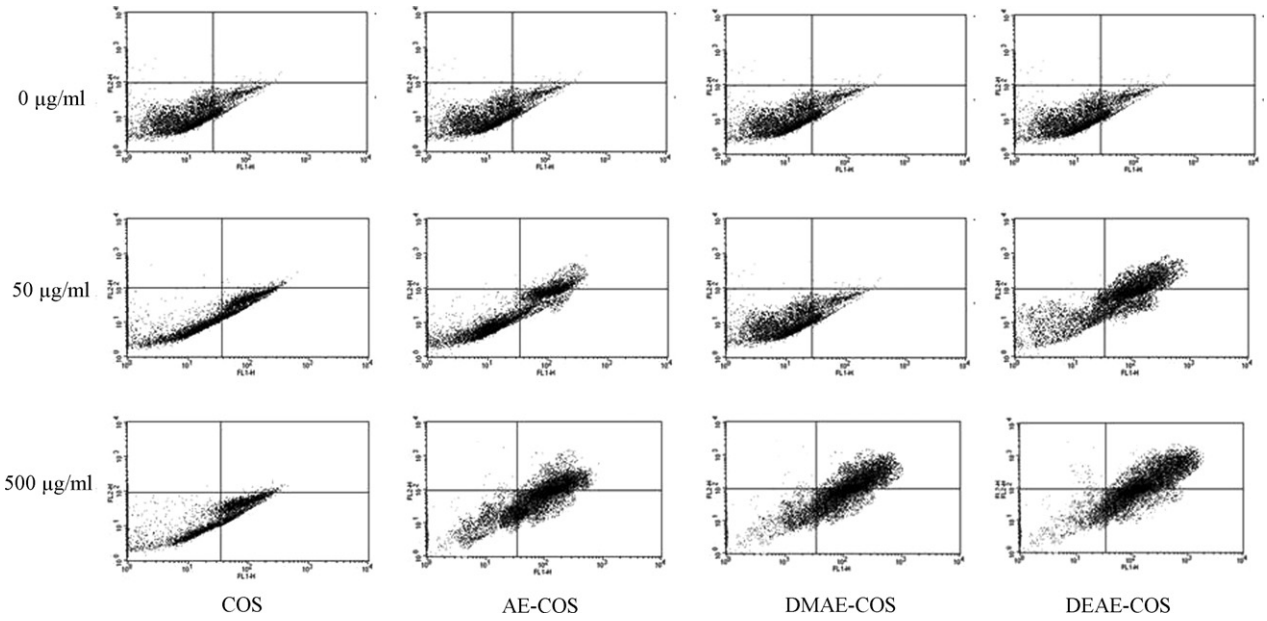

Fig. 5. Detection of Annexin V staining by FACS in AGS cells treated with COS and aminoethyl-COS derivatives (AE-COS, DMAE-COS and DEAE-COS) for 24 h. In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, in the upper right are in late apoptosis, and cells in the upper left quadrant are necrosis.

when the cells were exposed higher concentration of the samples but the there was no significantly induction of necrosis. While cells were detected at lower right quadrant under exposure to higher concentration of COS, at that time cells were detected at upper right quadrant under higher concentration of aminoethylated COS. These results suggest that aminoethylation of COS enhance anti-proliferative effect of COS by decreasing effectiveness time.

3.5. Apoptotic effect on gene and protein expression levels

The change rates of Caspase-9, Bax, p53 and p21 as an important regulator involved in apoptosis were characterized to examine the apoptotic issues by Western blot (Fig. 6) and using RT-PCR (Fig. 7) analysis. While GAPDH was used as internal standard for RT-PCR, β-tubulin was used as internal standard for Western blot analysis. According to results of Western blot assay, aminoethylated COS derivatives induced the up-regulation for Caspase-9, Bax, p21 and p53 proteins levels in a dose-dependent manner. According to RT-PCR assay results, p21, p53, Bax and Caspase-9 mRNA expression levels complies with the results of Western blot assay results. These results suggested that COS and aminoderivatized COS derivatives induced apoptosis in AGS cancer cells by mitochondrial pathways, via the up-regulation of Bax expression and activation caspases. In comparison to mitochondria of normal cell, the mitochondria of cancer cells involves more negative charge on surface which can be

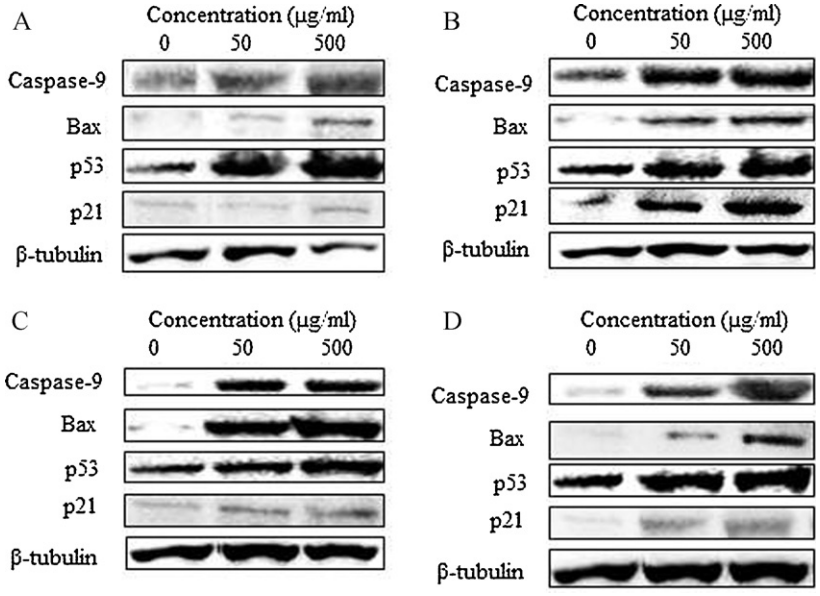

Fig. 6. Apoptotic effect of COS (A), AE-COS (B), DMAE-COS (C) and DEAE-COS (D) in apoptosis related genes. AGS cells were incubated with samples for 24 h and the expression levels of p21, p53, Bax and Caspase-9 m-RNA were detected using RT-PCR. GAPDH was used as an internal standard.

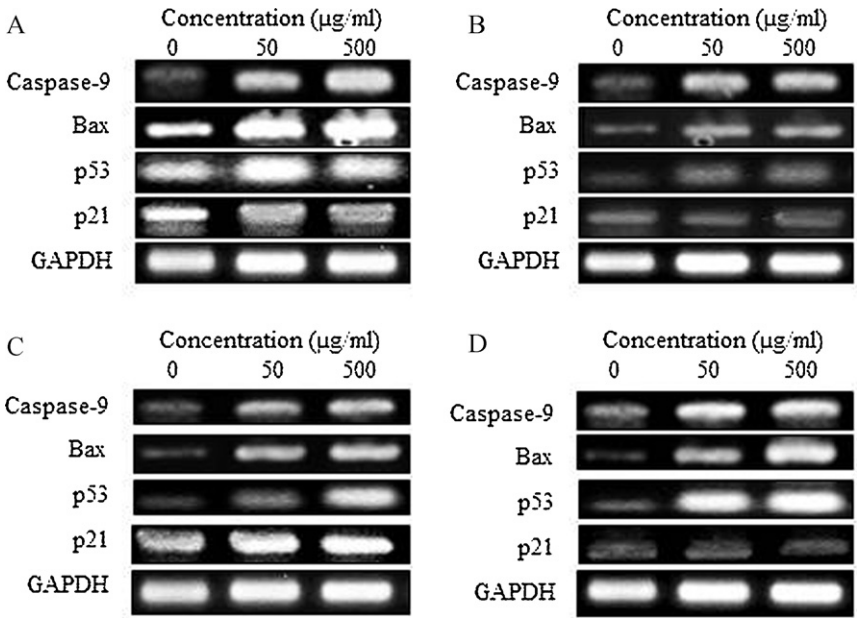

Fig. 7. Apoptotic effect of COS (A), AE-COS (B), DMAE-COS (C) and DEAE-COS (D) in apoptosis related proteins. AGS cells were incubated with samples for 24 h and the expression levels of p21, p53, Bax and Caspase-9 protein were detected using Western blot analysis. β-Actin was used as an internal standard.

targeted by positively charged molecules (Fantin, Berardi, Scorrano, Korsmeyer, & Leder, 2002). In this study, COS was modified by addition of aminoalkyl group at the C-6 position in order to develop the water-solubility and quantity of free amino group. It has been know that electric charge density due to the free amino groups in COS affects the cancer cell viability (Huang, Mendis, Rajapakse, & Kim, 2006; Qin et al., 2002). Replacement of amino group in COS caused to leading to increment of cationic charge, which might induce electrostatic interactions with the cancer cell surface. In comparative analysis, the modified COSs exhibited similar anti-proliferative effects at concentration of 500 μg/ml. In case of treatment with 50 μg/ml of the modified COS, the anti-proliferative effect was in the order of DEAE-COS ≥ DMAE-COS > AE-COS. The upgrade of the anti-proliferative activity of COS with DEAE group might be due to hydrophobic interactions between DEAE group and cancer cell surface.

4. Conclusion

In this study, water-soluble aminoethylated COS derivatives, AE-COS, DMAE-COS and DEAE-COS, were synthesized using COS with lower molecular weight (below 1 kDa) and their anti-proliferative effects were evaluated in AGS human gastric cancer cells. Exposure of AGS cells to the aminoethylated COS derivatives induced the inhibition of cell proliferation in a dose-dependent manner. Among them, DEAE-COS exerted a higher anti-proliferative activity. These results suggest that aminoethyl addition to COS enhances its apoptotic activity on AGS human carcinoma cells and aminoderivatized COS derivatives would be useful candidates in cancer chemopreventive therapy.

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