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Inhibitory effect of aminoethyl-chitooligosaccharides on invasion of human fibrosarcoma cells

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ABSTRACT

Chitooligosaccharides (COS) have been reported to show a variety of biological efficacies such as antibacterial activity, anti-tumor activity and immune activity. The purpose of this study is to investigate the inhibitory effect of aminoethyl-chitooligosaccharides (AE-COS) synthesized from COS that were substituted hydroxyl groups with aminoethyl group at C-6 position on cell invasion of human fibrosarcoma cells. First of all, the effect of AE-COS on cell viability was observed using MTT assay. The cytotoxicity of AE-COS was increased in a dose dependent manner. The inhibitory effects of AE-COS on the activity and expression level of MMP-2 and MMP-9 related to invasion of cancer cells were examined using gelatin zymography and western blot. It was found that AE-COS above 20 μg/ml showed the inhibitory effect on the activity and expression of MMP-9. Furthermore, AE-COS at 20 μg/ml reduced the expression level of p50, a part of NF-κB, compared with phorbol-12- myristate-13- acetate (PMA) group. The available data let us hypothesize that AE-COS could provide chemoprevention as an inhibitor against cell invasion associated with metastasis.

1. Introduction

In recent years cancer has been reported to be a main disease leading to increase of human mortality. The cause of death caused by cancer is identified to be due to invasion and metastasis (Brown and Murray, 2015). In general, metastasis occurs when the unstable cells surrounding tumor in tissue environment. Accordingly, in order to treat cancer and prevent our body from tumor development, above all, it is very important to inhibit metastasis. The degradation of base membrane and extracellular matrix are required for cancer cells to get propagated into other tissues. The matrix metalloproteinases (MMPs) plays a key role in cell invasion and metastasis during process that cancer cells move into the surrounding tissues and organ by degrading base membrane and extracellular matrix. MMPs, Zn-dependent enzyme, that are essential enzyme for angiogenesis and metastasis have been studied as a target protein for treatment of cancer. Among them, MMP-2 and MMP-9 using gelatin among extracellular matrix proteins as a substrate are specifically involved in metastasis (Wiranowska et al., 2015). In addition, MMPs were reported to act on wound healing of base membrane and cell growth by expression of growth factors (Martins et al., 2013). Therefore, it is important that the expression level and activity of MMPs maintains in constant status in body. Tissue inhibitor of metalloproteinases (TIMPs) were known to regulate MMPs activity (Arpino et al., 2015). Among them, the activity of MMP-2 and MMP-9 are inhibited by TIMP-1 (Nam and Kim, 2013). There are also NF-κB and AP-1 transcription factors in nucleus that modulate the expression and activity of MMPs. NF-κB is composed of p50 and p65 subunits, and AP-1 is consisted of c-fos sand c-jun subunits. NF-κB and AP-1 promote cancer activity and cell invasion and regulate inflammation induced by cytokines (Hsieh et al., 2014).

Among anti-cancer compounds, chitooligosaccharides (COS) have been known to exert antimicrobial activity, anti-cancer activity, immune activity and antioxidant activity. COS are readily soluble in water because of the presence of free amino groups in D-glucosamine units to shorter chain lengths (Lodhi et al., 2014). Aminoethyl-chitooligosaccharides (AE-COS) used in this study is a previously synthesized compound by our group that the hydroxyl group at C-6 is replaced by amino ethyl group to increase a biological activity. Our previous studies reported that the AE-COS exert an apoptotic effect of cancer cell and inhibitory effects on angiotensin converting enzyme (ACE) activity, oxidative stress and inflammation (Karagozlu et al., 2012; Ngo et al., 2012; Ngo et al., 2008). Therefore, we investigate whether AE-COS can inhibit the

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expression and activity MMPs that play a key role in cell invasion associated with metastasis in human fibrosarcoma cells

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin–EDTA, penicillin/streptomycin/amphotericin (10,000 U/ml, 10,000 g/ml, and 2500 g/ml, respectively) and fetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (NY, USA). HT1080 cells were purchased by ATCC. MTT reagent, agarose, and other materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA). AE-COS were synthesized by chitooligosaccharides (COS) with molecular weight 800–3000 Da and 90% of deacetylation by grafting 2-chloroethylamino hydrochloride at C-6 position. The synthetic product (95% purity) has 800.79–4765 Da of molecular weight and is well soluble in water and was designated as AE-COS by our group in previous study (Ngo et al., 2008).

2.2. Cell line and culture

Cell lines were separately grown as monolayers at 5% CO_2 and 37 °C humidified atmosphere using appropriate media supplemented with 5% fetal bovine serum, 2 mM glutamine and 100 g/ml penicillin–streptomycin. DMEM was used as the culture medium for HT1080 cell line. Cells were passaged 3 times a week by treating with trypsin–EDTA.

2.3. MTT assay

Cytotoxic levels of AE-COS on HT1080 cells were measured using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described by Hansen et al. (Hansen et al., 1989).

2.4. Gelatin zymography

Activation of MMPs were determined by zymography as described previously (Kang et al., 2008) in the presence or absence of AE-COS. Conditioned medium containing same amount of total proteins was loaded into each well of polyacrylamide gels containing 1.5 mg/ml gelatin and electrophoresed under non-reducing conditions. Depending on the purpose, different protein amounts for each experiment were used for gelatin zymography analysis. Gelatinolytic bands were observed as clear zones against the blue background and the intensity of the bands was estimated using ImageMaster Software (Amersham Pharmacia Bioscience, NJ, USA).

2.5. Extraction of nuclear proteins

After appropriate treatment of nuclear protein was extracted the cells treated with AE-COS. Briefly, the cells were harvested with 1 ml of ice-cold phosphate-buffered saline (PBS) and centrifuged for 1 min at 5000 rpm at 4 °C. The cell pellet was lysed with 0.4 ml of buffer A containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF), for 15 min on ice. Then, 25 ml of 10% Nonidet P-40 solution was added and the samples were vortexed for 15 s before centrifuging at 15,000 rpm for 5 min at 4 °C. The pellet was washed once with 0.5 ml of buffer A and re-suspended in 50 ml of buffer B, which was composed of 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. The lysed nuclei were left on ice for 30 min and then centrifuged at 15,000 rpm for 5 min at 4 °C. The nuclear protein concentration was determined by the DC Protein Assay (BioRad, Hercules, CA, USA). Nuclear extracts were stored at −80 °C until use.

2.6. Western blot analysis

Western blot analysis was performed according to standard procedures. Cells treated with different concentrations of AE-COS were lysed with RIPA lysis buffer (Sigma Chemical Co. St. Louis, MO, USA). Cell lysates were resolved on a 4–20% Novex®gradient gel (Invitrogen, USA), electro-transferred onto a nitrocellulose membrane and blocked with 10% skim milk. The primary antibodies including anti-MMP-9, anti-MMP-2, anti-TIMP-1 and anti-β-actin and their secondary antibodies (Santa Cruz BiotechnologfInc., CA, USA)were used to detect respective proteins using a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, NJ, USA) according to the manufacturer's instructions. Protein bands were visualized using AlphaEase®gel image analysis software (Alpha Innotech, CA, USA).

2.7. Analyses of protein expressions of MMP-9 and NF-κB using immunofluorescence staining assay

HT1080 cells were seeded in slide chamber and were incubated overnight at 37 °C. Then, cells were treated with AE-COS and stimulated with PMA as negative control. After 24 h of incubation, cells were fixed with 10% formalin for 15 min at room temperature followed by permeabilization with PBS containing 0.5% tween 20 (0.5% PBS T-20) and washing three times by 0.1% PBS T-20. The cells has preconditioning process with 5% Donkey normal serum and immunofluorescence staining with primary antibodies (MMP-9, NF-κB) (1:500) for 24 h at room temperature. After, the cells were then washed with 0.1% PBS T-20 three times for 5 min, respectively and treated secondary antibodies (donkey anti- rabbit conjugated FITC, donkey anti-mouse conjugated CY3) (FITC 1:200, CY3 1:400) at room temperature for 1 h. The cells were then washed with 0.1% PBS T-20 three times and PB once for 5 min, respectively. Finally, the slide was spread by DAPI solution and examined using iRiS™ Digital Cell Imaging System (Logos Biosystems, Annandale, US).

2.8. In vitro cell invasion assay

Cell invasion assay was performed according to the protocol from Chemicon®. The Cell Invasion Assay Kit (ECM550) utilizes an Invasion Chamber, which consists of a 24-well tissue culture plate and 12 cell culture inserts. The inserts contain an 8 lm pore size polycarbonate membrane, over which a thin layer of ECMatrix solution is applied (ECM layer occludes the membrane pores, blocking non-invasive cells from migrating through; in contrast, invasive cells migrate through the ECM layer). HT1080 cells in 300 μl of serum-free media were added to each insert and 500 μl of media containing 10% fetal bovine serum (chemoattractant) was added to the lower chamber. As soon as cells adhered to insert, cells were treated with AE-COS and stimulated with VEGF. Chambers were then incubated in 5% CO_2 and at 37 °C for 24–72 h. Non-invading cells as well as the ECM gel layer were removed using a cotton-tipped swab. On the contrary, the invasive cells on lower surface of the membrane were stained by dipping inserts in a staining solution for 20 min. Then, quantitate by dissolving stained cells in 10% acetic acid and transfer a consistent amount of the dye/solute mixture to a 96-well plate for colorimetric reading of OD at 560 nm.

2.9. Statistics

Data were analyzed using Student's *t* test for paired data (comparison with control group) and MEGFL. Data are given as means