Pre-treatment with tannic acid inhibits the intracellular IL-8 production by chitosan in a human oral epithelial cancer cell line

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Abstract: Chitosan stimulates the fibroblastic, endothelial, and intestinal epithelial cells to release chemotactic inflammatory cytokines, especially interleukin 8 (IL-8). Tannic acid (TA) has a variety of biological activities, such as anti-inflammatory, anti-viral effects. However, the pre-treatment of TA on chitosan polymer-induced IL-8 production from epithelial cells has not been well studied. This study focused on the effect of TA on chitosan polymer-induced IL-8 production in the oral mucosal epithelium as estimated by HSC-2 cells established from a human oral squamous cell carcinoma. The intracellular IL-8 production was measured by flow cytometry. The chitosan polymer-induced production of IL-8 decreased after pre-treatment with TA. IL-8 mRNA was measured by real-time PCR, and its mRNA expression level was reduced after TA pre-treatment. Antibody-based assays were used to investigate the effect of chitosan and TA on the activation of mitogen-activated protein kinases (MAPKs). These investigations showed the pre-treatment effects of TA on IL-8 production to be mainly mediated through the inhibition of the p38 MAPK pathways. The present study demonstrated that TA pre-treatment reduced the intracellular IL-8 production in HSC-2 cells via the MAPK signaling pathways. These findings indicate that the combined application of TA and chitosan may be clinically beneficial for the inflammatory lesions of the oral mucosa.

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Key words: chitosan polymer, tannic acids, IL-8, oral epithelial cell line, MAPK pathways

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Introduction

Chitosan has a variety of biological activities and is advertised as a healthy food that is effective in the improvement and/or care of various disorders, such as arthritis, cancer, diabetes, hepatitis, etc (1-2). It also accelerates wound healing, and helps to achieve a pleasant-looking skin surface and prevents excess scar formation (3-4).

Chitosan has been widely used as an effective medicament in various fields of medicine and dentistry (5-6). In endodontics, it can be used as an anti-inflammatory root canal dressing material for periapical lesions (7). Chitosan has also been applied clinically as a dressing for oral mucosal wounds and as a tampon following the radical treatment of maxillary sinusitis. Chitosan stimulates the fibroblastic, endothelial, and intestinal epithelial cells to release chemotactic inflammatory cytokines, especially IL-8 (8-10). IL-8 is a major chemokine for neutrophils which are largely responsible for phagocytosis at the initial inflammatory sites. *Actinobacillus actinomycetemcomitans* plays

an important role in initiating inflammatory reaction through the release of IL-8 from gingival epithelial cells (11). The mRNA level of human IL-8 is elevated by live cultures of a clinical isolate of *Aggregatibacter actinomycetemcomitans* in cultured gingival epithelial cells from healthy individuals (12). The histological findings indicate that chitosan induces the migration of polymorphonuclear leukocytes and macrophages in the applied tissue at an early stage (13). Therefore, the initial inflammatory reactions must be overcome before the chitosan can be clinically applied to gingival wounds.

Tannic acid (TA) has anti-oxidant, anti-inflammatory, and anti-carcinogenic properties (14-17). TA reduced UVB induces IL-18 production from human keratinocyte cell line, HaCaT, and TA regulates the p38 mitogen-activated protein kinase (MAPK) and ERK (18). Cranberry components (polyphenolic rich fruit) inhibit IL-6, IL-8, and prostaglandin E2 produced by lipopolysaccharide-activated gingival fibroblasts (19). However, the anti-inflammatory properties of TA on chitosan-induced IL-8 production from epithelial

cell have not been well studied.

The aim of this study was to investigate the effect and the sub-cellular mechanism of TA on the production of IL-8, an inflammatory mediator, by HSC-2 cells stimulated by chitosan polymer. This model system may therefore suggest a new application of TA to suppress the initial inflammation by chitosan dressing on the oral mucosa.

Materials and methods

Preparation of chitosan and TA

The chitosan polymer used in this study was supplied by Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The degree of deacetylation of chitosan was 88.8%. Its molecular weight was approximately 580 KDa. A 0.5% (w/v) solution of chitosan polymer was prepared by dissolving chitosan polymer powder in 0.4% (v/v) acetic acid. The completely dissolved solution was neutralized to pH 7.4, and was then filtered using a 0.45 µm filter. A 0.4% acetic acid solution (pH 7.4) was used as a control solution.

The TA was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The molecular weight of the TA used in this study was about 250-4,000 Da. A 0.5% (w/v) solution was prepared by dissolving powdered TA in ultra-pure water. The completely dissolved solution was neutralized to pH 7.4 and was then filtered using a 0.2 µm filter.

Cell culture

A cell line, HSC-2 established from a human oral squamous cell carcinoma, was purchased from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). The HSC-2 cells were seeded at 5×10^5 cells per 100 mm culture dish and maintained in 9 ml of Eagle's alpha minimum essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were cultured in a humidified atmosphere of 95% air with 5% CO₂ at 37°C. The medium was replaced every 3 days. HSC-2 cells between passages 6 and 9 were used for this experiment.

IL-8 production assay

HSC-2 cells were seeded in a 35 mm culture dish at a density of 5×10^5 cells in α -MEM. A 0.5% (w/v) solution of chitosan polymer was diluted to prepare 0.2, 0.1, 0.05, 0.025, 0.01, and 0.005% (w/v) solutions. After the cells were seeded, 2 mM of monensin (EMD Chemicals Inc., Gibbstown, NJ, USA) for blocking the protein transportation from the Golgi body to the cytoplasm and the chitosan polymer solution was added to the medium. After 2 to 8 hrs of culture with or without chitosan, cells were scraped off from the dishes with a rubber policeman. In order to assess the inhibitory effect of TA in chitosan polymer-induced IL-8 production, HSC-2 cells were cultured with 0.0005%,

Table 1. Effects of chitosan polymer and tannic acid (TA) on relative levels of phosphorylation of various kinases.

protein		phosphorylation sites	ratio ^a	
full name	abbreviation		unstimulated vs. chitosan	TA+chitosan vs. chitosan
extracellular signal-regulated kinase 1	ERK1	T202/Y204	1.1	0.9
extracellular signal-regulated kinase 2	ERK2	T185/Y187	1.8	0.9
Jun N-terminal protein-serine kinases 1	JNK1	T183/Y185	1.3	0.9
Jun N-terminal protein-serine kinases 2	JNK2		1.6	0.9
Jun N-terminal protein-serine kinases 3	JNK3	T221/Y223	1.3	0.8
Jun N-terminal protein-serine kinases pan	JNKpan	T183/Y185 T221/Y223	1.8	0.9
mitogen- and stress-activated protein kinase 2	MSK2	S360	1.4	0.6
p38 Mitogen-activated protein kinase α	p38 α	T180/Y182	2.2	0.8
p38 Mitogen-activated protein kinase β	p38 β	T183/Y185	1.6	1.0
p38 Mitogen-activated protein kinase γ	p38 γ		1.5	0.7
p38 Mitogen-activated protein kinase δ	p38 δ	T180/Y182	1.6	0.8
ribosomal S6 kinase 1	RSK1	S380	1.3	0.9
ribosomal S6 kinase 2	RSK2	S386	1.1	0.8
glycogen synthase kinase-3 α/β	GSK-3 α/β	S21/S9	1.7	0.5
glycogen synthase kinase-3 β	GSK-3 β	S9	1.6	0.8
heat shock protein 27	HSP27	S78/S82	0.7	1.0
serine-threonine kinase 1	Akt1	S473	1.8	0.6
serine-threonine kinase 2	Akt2	S474	1.1	0.7
serine-threonine kinase 3	Akt3	S472	1.1	0.7
serine-threonine kinase pan	Akt pan	S473/S474/S472	1.2	0.6

^aConfirmed by densitometric value. Data are from duplicate samples.

Fig. 1. The effect of treatment with chitosan polymer on the production of IL-8 in HSC-2 cells. The data are the mean $\pm$ standard deviations (SD) of the triplicate assays at 2, 4, 6, 8 hrs. **P*-value of < 0.05 compared to 4 hrs of incubation group. The largest production of IL-8 was shown after the incubation with 0.025% of chitosan.

0.001% and 0.002% (w/v) of TA for 2 hrs before the application of chitosan. After the treatment, phosphate-buffered saline(-) was added to the scraped cells, which were centrifuged at $300 \times g$ for 5 min. Cell pellets were treated by IntraPrep Reagent 1 (Beckman Coulter Inc., Fullerton, CA, USA) at room temperature for 15 min to fix the cells. After the treatment, the cells were collected in PBS(-) and centrifuged at $300 \times g$ for 5 min. The cell pellet was treated by IntraPrep Reagent 2 (Beckman Coulter) at room temperature for 5 min to regulate the membrane's permeability. After the treatment, FITC-labeled anti-human IL-8 antibodies (R&D Systems Europe Ltd., Abingdon, UK) or anti-mouse IgG1-FITC as an isotype control were added and incubated at room temperature for 15 min in a dark area. After adding PBS(-), the reacted sample was centrifuged at $300 \times g$ for 5 min. The cell pellet was suspended in PBS(-) supplemented with 0.5% (w/v) paraformaldehyde. 10,000 cells/sample were analyzed using EPICS XL flow cytometer (Beckman Coulter), which was equipped with an argon laser, at a wavelength of 488 nm.

Isolation of RNA

HSC-2 cells were seeded in a 60 mm culture dish at a density of 5×10^5 cells and cultured for 2 or 4 hrs in α -MEM containing 10% FBS supplemented with 0.025% (w/v) chitosan polymer, or chitosan polymer and 0.001% (w/v) TA. The cells were rinsed twice with PBS(-) and scraped off from the dishes. The total RNA was prepared using an AtlasTM Glass Total RNA Isolation kit (Takara Bio Inc., Otsu, Japan), according to the instructions provided by the manufacturer.

RT-PCR

HSC-2 cells were seeded in a 60 mm culture dish at a density of 5×10^5 cells and cultured for 2 or 4 hrs in α -MEM containing 10% FBS supplemented with 0.025% (w/v) chitosan polymer, or 0.001% (w/v) of TA + chitosan polymer. In order to investigate the effects of the chitosan polymer-induced IL-8 production via the MAPK signaling

pathways, HSC-2 cells were pretreated with either an upstream inhibitor of extracellular signal-regulated kinase (ERK) (PD98059) or p38 MAPK (SB203580) at a concentration of 10 μ M for 2 hrs prior to the chitosan exposure. These specific inhibitors were purchased from EMD Chemicals. After the HSC-2 cells were incubated, they were rinsed twice with PBS(-). Total RNA was extracted using Trizol[®] reagent (Life Technologies Inc., Carabid, CA, USA), according to the manufacturer's instructions. cDNA synthesis was performed using a SuperScriptTM First-Strand Synthesis System (Life Technologies) and an oligo dT according to manufacturer's instructions. cDNA obtained from 400 ng of the total RNA was used for one PCR. For amplification of IL-8 via PCR, the forward primer sequence was 5'-ATGAC TTCCA AGCTG GCCGT GGCT-3', and the reverse one was 5'-TCTCA GCCCT CTTCA AAAAC TTCTC-3' (20). For glyceraldehyde phosphate dehydrogenase (GAPDH), the forward primer sequence was 5'-CGGAG TCAAC GGATT TGGTC GTAT-3', and the reverse one was 5'-ACCCT TCTCC ATGGT GGTGA AGAC-3' (20). A master mix of the following PCR reaction components was prepared to the indicated quantity: 9.1 μ l of water, 0.3 μ l of diluted reference dye, 0.2 μ l of forward and reverse primers and 10 μ l of Brilliant[®] SYBR[®] Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). The above-mentioned reaction was placed in a 0.2 ml tube and 0.2 μ l of cDNA was added as the PCR template for amplification via PCR. The PCR sequence protocol was as follows: an initial denaturation at 95°C for 10 min; 30 cycles were conducted, each cycle consisting of 15 sec of denaturation at 94°C, 18 sec of annealing at 55°C and 45 sec of extension at 72°C; and a final step of 72°C for 1 min. The amplification and data acquisition were carried out using an Mx3000P QPCR machine (Stratagene).

The quantification was accomplished by determining the threshold cycle (the second derivative of the resulting fluorescence curve) at which the amplicon is detected during the PCR and comparing this to the standard curve calculated from the parallel quantification reactions. These calculations

Fig. 2. The effect of pre-treatment with tannic acid (TA) for 2 hrs on the production of chitosan (0.025%, 4 hrs)-induced IL-8 in HSC-2 cells. The data are the mean $\pm$ SD of the triplicate assays. **P*-value of < 0.05 compared to the untreated control. An effective inhibition was observed at a 0.001% of TA.

were done with the comparative quantification method using the Stratagene form. These data were normalized by GAPDH.

Antibodies assay for phosphorylation of MAPKs

HSC-2 cells were seeded in 100 mm culture dish at a density of 5×10^5 cells. After 10 days of culture, HSC-2 cells sub-confluent in dish were pre-treated with 0.001% of TA for 2 hrs and were then treated with 0.025% chitosan polymer for 30 min. The cells were retrieved and lysed following the manufacturer's instructions.

The relative level of phosphorylation of various signal transduction kinases was analyzed using a human MAPK array kit (R&D) (21). Phospho-site specific biotinylated antibodies (Table 1) were used to detect twenty different kinds of phosphorylated kinases via streptavidin-HRP, and then the membranes were exposed to X-ray film and the detected signals were developed on film. Thereafter, each signal was quantitatively measured using a densitometric mode of ChemiImagerTM (Alpha Innotec Corp., San Leandro, CA, USA).

Statistical analysis

The statistical significance was determined by means of Fisher's PLSD test and the StatView software program (SAS Institute Inc., Cary, NC, USA). The results were considered to be statistically significant with a *P* value of < 0.05 .

Results

IL-8 production

Chitosan polymer stimulated the IL-8 production in HSC-2 cells (Fig. 1). The incubation of these cells with 0.025% (w/v) of chitosan polymer for 4 hrs induced the largest production of IL-8. The chitosan polymer-induced production of IL-8 from HSC-2 cells was reduced by a pre-treatment with TA (Fig. 2). This inhibition was dependent on the concentration of TA. Since the 0.002% of TA also inhibited the cell viability, thus the inhibitory effect of TA on the mRNA level was examined at 0.001%.

RT-PCR

The IL-8 mRNA expression level by chitosan polymer was reduced after 2 hrs of TA pre-treatment (Fig. 3). The extent of the inhibitory reaction was similar at 2 and 4 hrs of chitosan application. The 4 hrs of incubation was used for the next experiment, because cell damage did not occur by the longer application of chitosan.

Although there was no statistical difference, the pre-treatment of specific inhibitors for MAPK pathways, PD98059 or SB 203580, effectively reduced IL-8 mRNA expression level in comparison to the chitosan polymer group (Fig. 4). The inhibitory reaction was recognized at a similar level between TA and the specific inhibitor applications.

Antibodies assay for phosphorylation of MAPKs

The expression of various phosphorylated kinases in the chitosan polymer group or TA and the chitosan group compared to the control group is shown in Table 1. Fifteen kinases exhibited a difference ratio between chitosan polymer group and the unstimulated group of ≥ 1.2 . The ratios of ERK2, JNK1, JNK2, JNK3, JNK pan, MSK2, p38g, p38d, p38a, p38b, RSK1, GSK-3a/b, GSK-3b, Akt1,

Fig. 3. The effect of pre-treatment with a 0.001% of TA on the expression of IL-8 mRNA in HSC-2 cells stimulated by a 0.025% of chitosan for 2 hrs (a) and 4 hrs (b). The data are the means $\pm$ SD of triplicate assays. **P*-value of < 0.05 compared to the controls (unstimulated and chitosan polymer groups). The similar inhibition of IL-8 mRNA was observed by both hours of incubation.

Fig. 4. The effect of pre-treatment for 2 hrs with PD98059 or SB203580 on the expression of IL-8 mRNA in HSC-2 cells stimulated by chitosan for 4 hrs. The data are the mean $\pm$ SD of the triplicate assays. Both inhibitors induced the effective inhibition of IL-8 mRNA.

and Akt pan were 1.8, 1.3, 1.6, 1.3, 1.8, 1.4, 2.2, 1.6, 1.5, 1.6, 1.3, 1.7, 1.6, 1.8, 1.2, respectively. Twelve kinases exhibited a difference ratio between TA + the chitosan group and the chitosan group of ≤ 0.8 . The ratios of JNK3, MSK2, p38g, p38d, p38a, RSK2, GSK-3a/b, GSK-3b, Akt1, Akt2, Akt3, and Akt pan were 0.8, 0.6, 0.7, 0.8, 0.8, 0.8, 0.5, 0.8, 0.6, 0.7, 0.7, 0.6, respectively.

Discussion

The information thus obtained is a fairly accurate representation of the *in vivo* situation as cytokine production profiles can be assessed in freshly obtained cells that have been activated *in vitro* for several hours (22). The detection of intracellular cytokine, such as observed in the present study by flow cytometry, is a rapid, easy and semi-quantitative assay for screening homogenous cells in order to determine their cytokine pattern (23).

Pre-treating HSC-2 cells with either PD98059 or SB203580 before the chitosan application reduced the IL-8 mRNA expression level. This finding strongly indicates that chitosan-induced IL-8 production in epithelial cells is mediated through MAPK pathways. In addition, chitosan regulates epithelial and mesenchymal cells via stimulation similar to the regulation by ultraviolet radiation (9), IkBa ubiquitination (18), and lipopolysaccharides (19). The inflamed gingival epithelium is severely infiltrated with leukocytes around the periodontal pocket, accompanied by an elevated expression of IL-8 (24). Thereafter, one of epithelial cell line, HSC-2 was used in this study because cysteine proteinases from *Porphyromonas gingivalis*, or gingipains which are key virulence factors of periodontal diseases preferentially suppress IL-8, thus resulting in the attenuation of the cellular recognition in HSC-2 cells of bacteria (25). Chemokines, such as IL-8, form the first line of host defense for lysosomal enzymes and superoxide anion generation (26), thus indicating that this mechanism plays an important role in innate immunity.

The combined biological application of chitosan and TA is mainly for TA adsorption and TA release into or from chitosan, respectively (27-28). This study is the first to report that TA has an inhibitory effect on chitosan polymer-induced IL-8 production, which may be beneficial for clinical applications. This means that the mild inhibition of IL-8 production could thus be achieved at the initial inflammatory sites of the oral epithelium. The chitosan application to the gingival abscess and periodontal pockets has been clinically carried out for wound healing (personal communications).

Natural products have been investigated more thoroughly than chemical agents as promising agents to prevent inflammatory diseases, in general (29-31), and plaque-related oral lesions, such as caries (32-33). There are many natural nitrogen-containing organic compounds that possess biological activities. For example, the amino group containing nitrogen is exposed to various degrees of deacetylation of chitin. Chitosan is a biocompatible and biodegradable material. After the chemotactic accumulation of inflammatory cells through IL-8 production, chitosan plays a role in the reorganization of the extracellular matrix during the final stage of wound healing (34-36). These phenomena are thought to also occur in the oral mucosa. Therefore, the inhibition of the initial inflammatory reaction after applying chitosan is beneficial with respect to its clinical application. The TA-induced inhibitory reaction to epithelial cells also induces the reduction of cytokine production by gingival fibroblasts.

In addition, the present study investigated whether or not a bioactive natural product, TA, regulates and affects the p38 MAPK and ERK signaling pathways. The TA group showed that IL-8 mRNA expression of TA and the chitosan group was reduced in comparison to the chitosan polymer group. In this study, the antibody assay for the phosphorylation of MAPKs showed that the pre-treatment effects of TA on the chitosan-induced IL-8 production were mediated mainly through the inhibition of the p38 MAPK pathways. The family of p38 MAPKs (p38a, p38b, p38d, and p38g) are mainly activated by cellular stress and inflammatory cytokines, although they also regulate non-stress-related functions such as proliferation, differentiation and development (37-39). GSK-3b was originally identified as a negative regulator of glycogen synthase in insulin-responsive tissues (40). GSK-3 is ubiquitously expressed and it demonstrates basal catalytic activity in cells. Following the stimulation with agonists, the inactivation of GSK-3 occurs through the phosphorylation of several residues and this is mediated by a variety of upstream kinases. In this regard, one of the most studied GSK-3b upstream kinases is Akt (41-42). The present antibody study clearly supports this intimate relationship between Akt and GSK. MSK1/2s are nuclear kinases that act downstream of the MAPK/ERK pathways (43-44). Recently, these kinases have been shown to be mandatory for the stress-induced phosphorylation of histone H3-Ser¹⁰ and for the transcriptional activation of several immediate early genes (45). The present data demonstrate the signal transduction pathways, from upstream

to downstream, through ERK, p38, Akt, GSK, and MSK. Furthermore, the sub-cellular inhibitory effect of TA on the chitosan polymer-induced IL-8 production in cultured epithelial cells is mediated via the Akt-rich signal pathways.

Sponge-type chitosan may be applied to wound healing in the oral mucosa, gingiva, and periodontal pocket. The chitosan monomer acts as a biocompatible, stable medicament even at the initial stage of wound healing because of the minimum inflammatory reaction in comparison to the application of the chitosan polymer (46). However, as the physical properties of chitosan monomer are very poor and easily dissolvable, it could not be used as a scaffold and as a carrier material. Therefore, if the initial chemotactic activity of the chitosan polymer is inhibited, its clinical application such as wound dressing may be popularized more safely.

In conclusion, the present study demonstrates that pretreatment with TA may reduce the intracellular IL-8 production in HSC-2 cells via the MAPK signaling pathways. These findings indicate that the combined application of both TA and chitosan may be clinically beneficial treatment for inflammatory lesions in the oral mucosa.

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