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Anti-inflammatory Activity of *Codium fragile* in Macrophages Induced by Peptidoglycan

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Abstract – To fine out the anti-inflammatory activities of the *C. fragile*. and its mechanism were investigated in macrophages induced by Peptidoglycan (PGN). Treatments of macrophages with 100 ug/ml of ethanol extract of *Codium fragile* (EECF) inhibited PGN-induced IL-6, NO and PGE_2 production in a dose-dependent manner as well as expression of iNOS and COX-2. EECF inhibited PGN-induced extracellular signal-regulated kinase (ERK) 1/2, JNK 1/2 and p38 MAPK phosphorylation, which suggests that EECF inhibits IL-6 and NO secretion by blocking MAPKs phosphorylation. These findings may help elucidate the mechanism by which EECF modulates RAW 264.7 cell activation under inflammatory conditions.
Keywords – Ethanol extract of *Codium fragile* (EECF), anti-inflammatory activities

Introduction

Sea staghorn (*Codium fragile*) is a siphonous marine green alga belonging to the family Codiaceae (order Siphonales, class Chlorophyceae). In China, it is eaten as food by some of the people along the coastlines and is used as anticancer, antiviral, antipyretic, and helminthic agents in Chinese traditional medicine (Zhang *et al*., 1994). It is also consumed as edible one and anthelmintic property in Korea. However, there is yet no report regarding *C. fragile* inducing anti-inflammatory effect in macrophage cells. Understanding the underlying mechanism related to the inhibition of inflammatory by *C. fragile* will benefit the potential new source of drug for the treatment of inflammatory diseases.

Bacteria stimulate the innate immune system of a host and the release of inflammatory molecules such as cytokines and chemokines as a response to infections (Medzhitov *et al*., 1998; Hoffmann *et al*., 1999). Peptidoglycan (PGN), a cell wall component of Gram-positive bacteria, is an alternating β-linked *N*-acetylmuramyl and *N*-acetylglucosaminyl glycan whose residues are cross-linked by short peptides(Bone *et al*., 1994; Ulevitch *et al*., 1995, Nakanishi *et al*., 2010).

Macrophages play an important role in host defenses against noxious substances, and are involved in a variety of disease processes, including autoimmune diseases, infections, and inflammatory disorders (Pierce *et al*., 1990). An inflammatory stimulus such as PGN can activate macrophages to produce a variety of pro-inflammatory cytokines like IL-6, as well as other inflammatory mediators, including prostaglandin (PG) E_2 and nitric oxide (NO), which are synthesized by cyclooxygenase (COX)-2 and inducible NO synthase (iNOS), respectively. The following three well-defined mitogen-activated protein kinases (MAPKs)-extracellular signal-regulated kinase (ERK) MAPK, p38 MAPK and c-Jun NH_2-terminal kinase (JNK) MAPK-have been implicated in the transcriptional regulation of the iNOS gene. Also, specific MAPK inhibitors suppress the expression of the iNOS gene (Chen *et al*., 1999; Chan *et al*., 1998; Kim *et al*., 2004).

Marine bioresources are known to be attractive as they sometimes contain new compounds showing several kinds of different bioactivities which are not possible in land plants. Screening of algal extracts for biologically active compounds began in the 1950s with simple

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antibiotic assays and expanded to include testing for products with antiviral, antibacterial, antifungal, anti-mitotic, and antitumorigenic activities (Zhu *et al*., 2006; Oh *et al*., 2008; Bennamara *et al*., 1999; Glombitza *et al*., 1989). Studies on antitumor effects of algal species have been reported by a number of researchers (Kwon *et al*., 2007; Cavas *et al*., 2006; Athukorala *et al*., 2006; Zhou *et al*., 2005).

Experimental

Plant material and extraction – *Codium fragile* was presented from the Dr. H.G. Choi (Faculty of Biological Sciences and Research institute for Basic Science). The *C. fragile* was air-dried in the dark at room temperature (RT) and then ground into a powder using a mechanical grinder. Approximately 500 g of the powdered materials was then in 1500 mL of ethanol for 7 day (room temperature). EtOH extract yield is 36.49 g. The extract was filtered (pore size, 0.45 µm) lyophilized, and kept at 4 ºC. The dried extract was then dissolved in phosphate buffered saline (PBS) in preparation for use. A Vouchermen (No.08-005) of the *C. fragile* has been deposited at the Herbarium of the College of Pharmacy, Wonkwang University, Iksan.

Reagents – PGN (derived from *Staphylococcus aureus*) was purchased from Fluka (Buchs, Switzerland). RPMI 1640, penicillin, and streptomycin were obtained from Hyclone (Logan, UT, USA). Bovine serum albumin was purchased from Sigma (St. Louis, MO, USA). COX-2, iNOS, p38, phosphorylated p38, ERK, phosphorylated ERK, JNK, phosphorylated JNK, β-actin, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-mouse IL-6 antibody and biotinylated anti-mouse IL-6 antibodies were purchased from BD Biosciences (San Jose, CA, USA). In addition, RNeasy Mini kit and QuantiTect Reverse Transcription kit were purchased from Qiagen (Hilden, Germany). Finally, IL-6, COX-2, iNOS and β-actin oligonucleotide primers were purchased from Bioneer Corp. (Daejung, Republic of Korea).

Cell culture – The murine macrophage cell line, RAW 264.7, was obtained from the Korea Research Institute of Bioscience and Biotechnology (Seoul, Republic of Korea) and grown in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/mL of penicillin/streptomycin sulfate. The cells were incubated in a humidified 5% CO_2 atmosphere at 37 ºC.

Enzyme-linked immunosorbent assay (ELISA) – Cells were seeded at 5×10^5/mL per well in 24-well tissue culture plates and pretreated with various concentrations of EECF (100 µg/mL or 50 µg/mL) for 30 minutes before PGN (30 µg/mL) stimulation. ELISA plates were coated overnight at 4 ºC with anti-mouse IL-6 antibody diluted in coating buffer and then washed three times with PBS containing 0.05% Tween 20. The nonspecific protein binding sites were blocked with assay diluent for at least 1 hour. Immediately, each sample and IL-6 standard were added to the wells. After incubation for 2 hours, a working detector was added and incubated for 1 hour. Accordingly, substrate solution was added to the wells and incubated for 30 minutes in the dark before the reaction was stopped with stop solution (2 *N* H_3PO_4).

Measurement of PGE_2 production – The RAW 264.7 cells were cultured in 24-well culture plates (5×10^5/mL). EECF (100 µg/mL or 50 µg/mL), PGN (30 µg/mL) was added to the culture medium and incubated at 37 ºC for 24 hours. The medium was collected in a microcentrifuge tube and centrifuged. The supernatant was decanted into a new microcentrifuge tube, and the amount of PGE_2 was determined by a PGE_2 Enzyme Immuno-Assay Kit according to the procedure described by the manufacturer.

Measurement of NO production – NO production was assayed by measuring the nitrite in the supernatants of cultured RAW 264.7 cells. The cells were seeded at 1×10^5/mL in 96-well culture plates. After preincubation of the RAW 264.7 cells for 18 hours, the cells were pretreated with EECF (100 µg/mL or 50 µg/mL) and stimulated with PGN (30 µg/mL) for 24 hours. The supernatant was mixed with anequal volume of Griess reagent and incubated at room temperature for 5 minutes. The concentrations of nitrite were measured by reading at 570 nm. Sodium nitrite ($NaNO_2$) was used to generate a standard curve.

Western blot analysis – Protein expression was assessed by western blot analysis according to standard procedures. The RAW 264.7 cells were cultured in 60-mm-diameter culture dishes (3×10^6/mL) and pretreated with various concentrations of EECF (100 µg/mL or 50 µg/mL). After 30 minutes or 1 hour, PGN (30 µg/mL) was added to the culture medium, and the cells were incubated at 37 ºC. After incubation, the cells were washed twice in ice-cold PBS (pH 7.4). The cell pellets were resuspended in lysis buffer on ice for 15 minutes, and cell debris was removed by centrifugation. According to the manufacturer's instructions. Equal amounts of protein (20 µg) were subjected to SDS-polyacrylamide gel electrophoresis.

The membrane was blocked with 5% nonfat milk in Trisbuffered saline with Tween 20 buffer. After blocking,

the membrane was incubated with primary antibodies for 18 hours. The membrane was then washed with Trisbuffered saline with Tween 20 and incubated with anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence (Amersham, Milan, Italy).

RNA Extraction and Reverse-Transcription PCR (RT-PCR) – The RAW 264.7 cells were cultured in 30-mm-diameter culture dishes (3×10^6/mL) and pretreated with various concentrations of EECF (100 μg/mL or 50 μg/mL). After 30 minutes, PGN (30 μg/mL) was added to the culture medium, and the cells were incubated at 37 °C. After incubation, the cells were washed twice in ice-cold PBS (pH 7.4). Total cellular RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany) and 1 μg of total RNA was reverse-transribed using QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany).

The primers used were as follows:

IL-6	5-CATGTTCTCTGGGAAATCGTGG-3	sense
	5-AACGCACTAGGTTTGCCGAGTA-3	antisense
COX-2	5-CACTCAGTTTGTTGAGTCATTC-3	sense
	5-GATTAGTACTGTAGGGTTAATG-3	antisense
iNOS	5-AGCCCAACAATACAAATGACCCTA-3	sense
	5-TTCCTGTTGTTTCTATTTCCTTTGT-3	antisense
β-actin	5-ATGAAGATCCTGACCGAGCGT-3	sense
	5-AACGCAGCTCAGTAACAGTCCG-3	antisense

Statistical analysis – The results are expressed as mean ± S.E values for the number of experiments. Statistical significance was compared between each treated group and the control and was determined by Student's *t*-tests. Each experiment was repeated at least three times and yielded comparable results. Values with $P < 0.05$ was considered significant.

Results and Discussion

Effects of EECF on PGN-induced IL-6 produceion and mRNA expression – Since EECF was found to inhibit the pro-inflammatory mediators, We investigated the effects of EECF on PGN-induced IL-6 production by ELISA and RT-PCR. Pretreatment of EECF reduced IL-6 production and IL-6 mRNA expression in a dose-dependent manner (Fig. 1).

Effects of EECF on PGN-induced PGE_2 production – To assess the effects of EECF on the PGN-induced PGE_2 production in RAW 264.7 cells, cell culture media were harvested and PGE_2 levels were measured. To examine

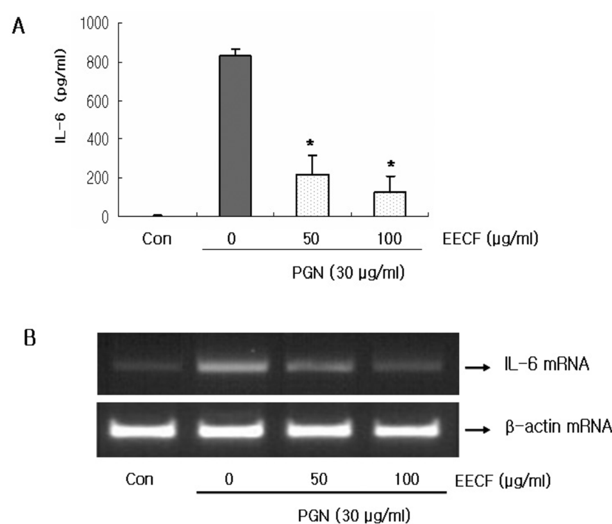

Fig. 1. Effects of EECF on PGN-induced IL-6 production and mRNA expression. (A) RAW 264.7 cells were pretreated with the indicated concentrations of CF for 1 hour before being incubated with PGN (30 μg/mL) for 24 hours. Production of IL-6 was measured by ELISA. (B) IL-6 mRNA was assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of EECF for 1 hour before being incubated with PGN (30 μg/mL) for 24 hours. Results are mean ± S.E. Statistical significance: $*p < 0.05$ vs. the PGN treated group.

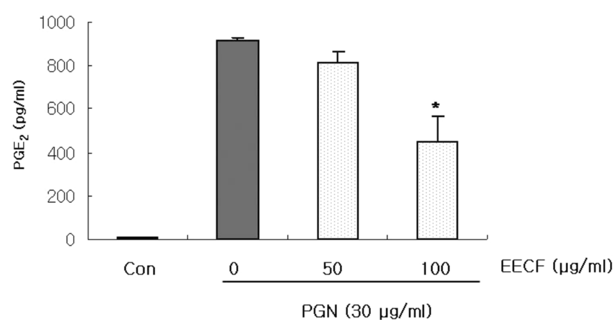

Fig. 2. Effects of EECF on PGN-induced PGE_2 production in RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentration of EECF for 1 hour before being incubated with PGN (30 μg/mL) for 24 hours. Control cells were incubated with the vehicle alone. $*P < 0.05$ vs. the PGN treated group.

whether EECF inhibits PGE_2 production, cells were pre-incubated with EECF for 1 hour and then activated with PGN (30 μg/mL) for 24 hours. As shown in Fig. 2, EECF inhibited the production of PGE_2 in dose-dependent manner.

Effects of EECF on PGN-induced COX-2 protein and mRNA expression – Western blot and RT-PCR analysis were performed to determine whether the inhibitory effects of EECF on PGE_2 is related to the modulation of the expression of COX-2. In unstimulated RAW 264.7 cells, COX-2 protein and mRNA were

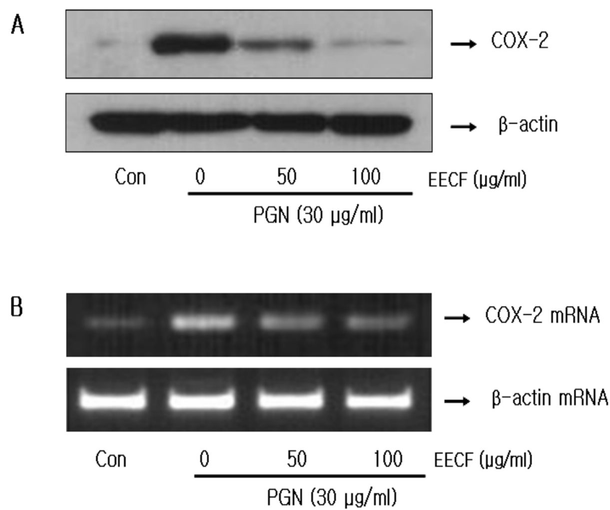

Fig. 3. Effects of EECF on PGN-induced COX-2 protein and mRNA expressions in RAW 264.7 cells. (A) RAW 264.7 cells were pretreated with the indicated concentrations of EECF for 1 hour before being incubated with PGN (30 µg/mL) for 24 hours. (B) COX-2 mRNA was assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of EECF for 1 hour before being incubated with PGN (30 µg/mL) for 24 hours.

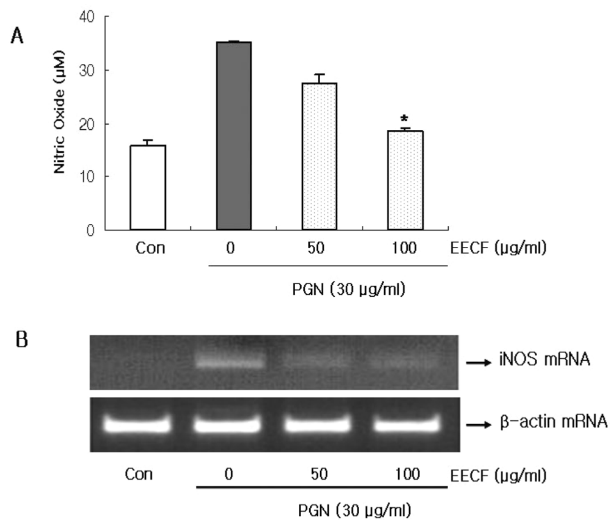

Fig. 4. Effects of EECF on PGN-induced NO production in RAW 264.7 cells. (A) RAW 264.7 cells were pretreated with the indicated concentrations of EECF for 1 hour prior to being incubated with PGN (30 µg/mL) for 24 hours. (B) iNOS mRNA was assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of EECF for 1 hour prior to being incubated with PGN (30 µg/mL) for 24 hours. Analysis of the β-actin mRNA was conducted in parallel to confirm equivalency of the cDNA preparation. $^*P < 0.05$ vs. the PGN treated group.

undetectable. However, in response to PGN, COX-2 is strongly expressed and EECF significantly inhibited COX-2 expression in a dose-dependent manner (Fig. 3A).

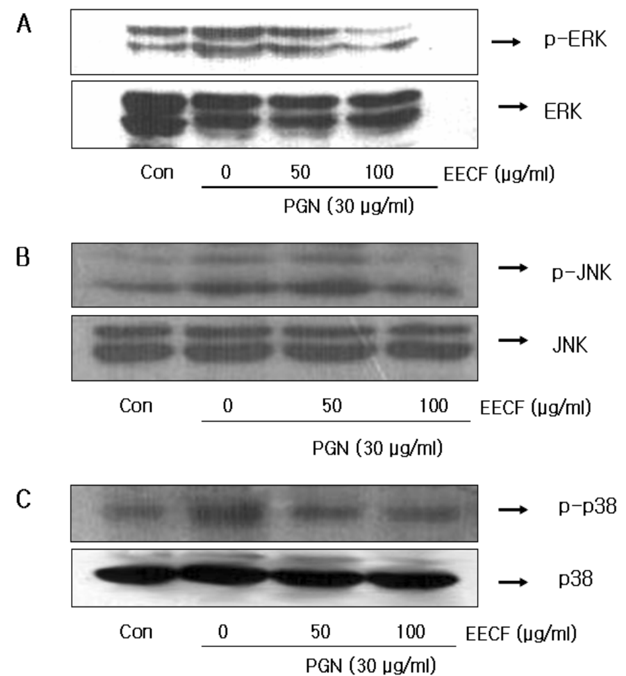

Fig. 5. Effects of EECF on phosphorylation of MAPKs in PGN stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of EECF for 30 min before being incubated with PGN (30 µg/mL) for 30 min.

RT-PCR analysis showed that COX-2 mRNA expressions related with their protein levels (Fig. 3B).

Effects of EECF on PGN-induced NO production and iNOS mRNA expression – To assess the effects of EECF on the PGN-induced NO production in RAW 264.7 cells, cell culture media were harvested and nitrite levels were measured. EECF treatment inhibited NO production dose-dependently in PGN stimulated RAW 264.7 cells. EECF inhibited NO production by 48.5% in PGN at high concentration, but only by a little at lower concentrations (Fig. 4A).

The effects of EECF on the PGN-induced expression of iNOS by RAW 264.7 cells was evaluated to identify the anti-inflammatory mechanism. iNOS was strongly expressed in cells that were treated by PGN, and RT-PCR analysis showed that iNOS mRNA expression was related to the nitrite levels (Fig. 4B).

Effects of EECF on the phosphorylation of MAPKs in PGN-stimulated RAW 264.7 cells – The MAPKs play critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses. We examined the effects of EECF on phosphorylation of MAPKs in PGN stimulated RAW 264.7 cells by Western blot analysis. EECF suppressed PGN stimulated activation of ERK 1/2 and p38 MAPK in

a dose-dependent manner (Fig. 5A, 5C). EECF inhibited ERK 1/2 and p38 MAPK activation, however, phosphrylation of JNK 1/2 was only affected by EECF treatment at high concentration (Fig. 5B).

Recently, many studies have evaluated the inhibitory effects of plant-derived anti-inflammatory agents *in vitro*. However, marine bioresources are not sufficiently investigated in term of their full therapeutical option. Marine algae produce various metabolites and have been recognized as promising targets in the search for biologically active compounds. So far, we have performed the screening studies on marine edible algae which could be important resources in the discovery of anti-inflammatory activities. *Codium fragile* is a green alga belonging to Codiales, and is widely distributed along the shore of Korea. *C. fragile* is one of familiar seaweeds and has been used as edible one from ancient times. Furthermore, Its use has been recorded in the treatment for enterobiasis, dropsy and dysuria in Oriental medical textbooks. *C. fragile* contains various compounds including clerosterol, codisterol, cholesterol(Rubinstein*et al*., 1974), tryptophan, indoleacetonitrile (Augier *et al*., 1970) and various polysaccharides (Yamamoto *et al*., 1974). However, no studies conducted to date have reported the mechanism by which the anti-inflammatory action of ethanol extract of *Codium fragile* (EECF) occurs.

In this study, we evaluated the pharmacological basis for EECF for the treatment of various inflammatory diseases. The effects of EECF on macrophage functions related to inflammation were investigated to verify possible mechanisms underlying its beneficial effects. We demonstrated that EECF inhibited PGN-induced pro-inflammatory mediators, including NO and PGE_2. To explore the mechanism of inhibition of NO and PGE_2 production in RAW 264.7 cells, the effects of EECF on the iNOS and COX-2 gene were examined. EECF inhibited the expression of COX-2 protein, COX-2 mRNA and iNOS mRNA in a dose-dependent manner, as assessed by Western blot analysis and RT-PCR, respectively. These results imply that EECF exerts its effects through the inhibition of the iNOS and COX-2 transcription.

NO is an important mediator in the inflammatory process and is produced at inflamed sites by iNOS. High levels of NO have been reported in a variety of pathological processes including various forms of inflammation, circulatory shock, and carcinogenesis (MacMicking *et al*., 1997; Ohshima *et al*., 1994; Szabó *et al*., 1995). Therefore, an inhibitor of iNOS might be effective as a therapeutic agent for inflammatory diseases (Koo *et al*., 2001). The results of this study showed that EECF inhibited PGN-induced NO production in RAW 264.7 macrophages. Taken together, these results indicate that EECF has a potent anti-inflammatory effect that occurs through inhibition of the expression of iNOS and NO production.

The MAPKs play a critical role in the regulation of cell growth and differentiation, particularly in response to cytokines and stress (Johnson *et al*., 2002). Therefore, the effects of EECF on the PGN-induced phosphorylation of ERK 1/2, JNK 1/2 and p38 MAPK were evaluated in this study. Interestingly, EECF inhibited PGN-induced phosphorylation of ERK 1/2 and p38 MAPK, but phosphorylation of JNK 1/2 was only inhibited at high concentration of EECF (Fig. 5). These findings suggest that ERK 1/2, JNK 1/2 and p38 MAPK are involved in the inhibition of pro-inflammatory mediators by EECF in RAW 264.7 cells.

In conclusion, We have shown that EECF inhibited PGN-induced IL-6, NO and PGE_2 productions, as well as iNOS and COX-2 expressions in macrophages and inhibited phosphorylation of ERK 1/2, JNK 1/2 and p38 MAPK. Thus, from this study, the anti-inflammatory effect of EECF can possibly be used as a therapeutic agent against Gram-positive bacteria. The fact that EECF has the effect on PGN, its anti-inflammatory activity will be greater against pathogens like MRSA. Therefore, it is possible after further research that EECF could be use against septicemia. Taken together, these findings indicate that EECF may represent a potential new source of drugs for the treatment of inflammatory disease.

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