Anti-Inflammatory Effects of *Gelidium amansii* Ethanol Extract on *Porphyromonas gingivalis* Lipopolysaccharide-Stimulated Human Gingival Fibroblasts through the Regulation of Nuclear Factor Kappa B/Activator Protein-1/Mitogen-Activated Protein Kinase Signaling Pathway

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**Objective:** Gingival inflammation is one of the main causes that can be associated with a lot of periodontal diseases. Among various periodontal pathogens, *Porphyromonas gingivalis* can be recognized as one of the main causes in the progression of the periodontal inflammation. In this study, the anti-inflammatory activity of *Gelidium amansii* ethanol extract (GAEE) and its molecular mechanism was investigated in *P. gingivalis* lipopolysaccharide (LPS-PG) stimulated human gingival fibroblast (HGF)-1 cells.

**Methods:** The concentration of nitric oxide (NO) and prostaglandin E₂ (PGE₂) production was estimated by biochemical analysis. Protein expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and their upstream signaling molecules were analyzed by Western blot analysis.

**Results:** LPS-PG induced NO and PGE₂ production as well as their corresponding enzymes, iNOS and COX-2, were significantly attenuated by GAEE treatment without cytotoxicity. The molecular mechanism was also investigated to determine whether this response was related to the inflammatory transcription factors, nuclear factor (NF)-κB and activator protein (AP)-1, or their upstream signaling molecules, mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)/Akt. Phosphorylated status of p65 and c-jun, each subunit of NF-κB and AP-1, was dose-dependently inhibited by GAEE treatment. In addition, GAEE treatment inhibited phosphorylation of extracellular regulated kinase (ERK) but did not give any effect on other MAPKs and PI3K/Akt signaling molecules.

**Conclusion:** Consequently, GAEE ameliorates LPS-PG-induced inflammatory responses by blocking NF-κB, AP-1 and ERK activation in HGF-1 cells. Therefore, GAEE may be utilized as a potential anti-inflammatory agent by modulating NO and PGE₂ production in the periodontium.

**Keywords:** *Gelidium amansii*, inflammation, mitogen-activated protein kinases, NF-kappa B, transcription factor AP-1

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Introduction

Inflammatory response refers to the reaction of tissues to repair the damage caused by physical damage or infection by viruses and microorganisms [1]. The periodontal disease represents a group of infectious inflammation caused by periodontopathic bacteria which destroy the tooth-supporting tissues, which can be classified as a gingival disease and periodontitis [2,3]. The gingival disease is the inflammation of the gingival tissues initiated by the accumulation of dental plaque. Periodontitis is the irreversible plaque-induced inflammation of the periodontal tissues, which can destroy the periodontal ligament and alveolar bone [4]. Among periodontal pathogens, Porphyromonas gingivalis, an obligatory anaerobic Gram-negative rod-shaped bacterium, is regarded as one of the main causes in the mediation and progression of the periodontal inflammation [5]. Lipopolysaccharide (LPS) is found to be an important pathogenic factor among many periodontopathic bacteria, which can activate the host inflammatory responses and disrupt the bone remodeling process [6]. The toll-like receptor (TLR)-4 is related to various microbial products for inflammatory mediators when LPS reaches to the cell surface [7]. Human gingival fibroblasts (HGFs) stimulated by LPS, overexpressed TLR4 can induce the production of proinflammatory mediators, such as interleukins (ILs), tumor necrosis factor-α, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2. Induced various proinflammatory mediators can initiate and amplify periodontal inflammation [8]. These inflammatory responses are closely related to periodontal tissue remodeling and the reduction of these inflammatory mediators can reduce the inflammatory responses in periodontium [9].

Many seaweeds are well-known for their various nutrients including polyphenols, carotenoids, vitamins, and phycobilins. Among them, Gelidium amansii is an edible red alga widely distributed in the shallow coast of Korea, China, Japan, and Taiwan. G. amansii has been used as an important food source and its agar product, prepared by boiling, filtering, cooling, and sweetening, has been consumed as one of the popular desserts in summer. In traditional medicine, G. amansii agar is known to have the effects on blood pressure, blood lipid, blood glucose, cancers and cardiovascular diseases [10]. Many researchers have tried to prove its various effects so far, and several studies have reported that G. amansii exhibits anti-oxidative, immunomodulatory, anti-tumor, and antiobesity effects [11-14]. Especially, the anti-inflammatory activity of G. amansii in the field of stomatology was not elucidated yet, we analyzed whether G. amansii ethanol extract (GAEE) can regulate P. gingivalis lipopolysaccharide (LPS-PG) induced inflammatory responses in HGF-1 cells.

Materials and Methods

1. Reagents

Dulbecco’s modified eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). LPS-PG was purchased from Invivogen (San Diego, CA, USA). GAEE was obtained from Jeju Biodiversity Research Institute (Jeju, Korea). Dimethyl sulfoxide and sodium dodecyl sulfate (SDS) were obtained from the Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against COX-2, phospho-p65, phospho-extracellular regulated kinase (ERK), ERK, phospho-c-Jun NH2-terminal kinase (JNK), JNK, phospho-p38, p38, and actin as well as horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) were purchased from Cell Signaling Technology (Boston, MA, USA).

2. Cell culture and treatment

The HGF-1 cell was obtained from American Type Culture Collection (CRL-2014; Rockville, MD, USA) and cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine.

3. Cell viability

Cell viability was determined using the WST-1 cell proliferation assay kit purchased from Daeil Lab. Service (Seoul, Korea). Cells were incubated with WST-1 for 1 hour and quantified by measurement at OD450.

4. NOS activity

HGF-1 cells were seeded in 6-well plate (2×10⁵ cells/well) and pre-incubated with various concentrations of agents for 2 hours. Then, 1 μg/ml of LPS-PG was added and incubated for 10 hours for NOS induction. For NOS activity measurement in cell lysates, HGF-1 cells were lysed by three times of freeze-thaw cycle in 0.1 ml of 40 mM Tris buffer (pH 8.0) containing 5 μg/ml of pepstatin A, 1 μg/ml of chymostatin, 5 μg/ml of aprotinin, and 100 μM phenyl methyl sulfonyl fluoride. Protein concentration was determined by Bradford assay. NOS enzyme activity was measured as previously described [15]. Briefly, 20 μg protein was incubated in 20 mM Tris-HCl (pH 7.9) containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM DTT, and 2 mM each of L-arginine and nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was performed in triplicate for 3 hours at 37°C in 96-well plate. Residual NADPH was oxidized enzymatically and the Griess reaction was performed.
5. Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) determination

PGE\textsubscript{2} concentrations in cell culture supernatants were measured by an EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions.

6. Western blot analysis

Cells (2×10\textsuperscript{6} cells/dish) in 100-mm dishes were pre-incubated with and without indicated concentrations of each sample for 2 hours, and then incubated with LPS-PG (1 μg/ml) for 18 hours. The cells were washed twice with phosphate buffer saline, scraped into 0.4 ml of protein extraction solution (M-PER; Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes at room temperature. The lysis buffer containing the disrupted cells was centrifuged at 13,000 × g for 10 minutes. Protein samples (25 μg) from each lysate were separated on a 10% SDS-polyacrylamide gel and electrotransferred to polyvinylidene fluoride or polyvinylidene fluoride membrane (Merck Millipore, Billerica, MI, USA). Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in tris-buffered saline with tween 20 solution. The reactions were then incubated at 4°C overnight with a 1:1,000 dilution of each primary antibody. After overnight incubation, the membranes were washed and then further incubated with a 1:1,000 dilution of HRP-conjugated anti-rabbit IgG for 2 hours at room temperature. The blots were developed with ECL developing solution (Thermo Fisher Scientific), and data were quantified using the Gel Doc EQ System (Bio-Rad, Hercules, CA, USA).

7. Statistical analysis

All data are expressed as the means±standard deviation. The statistical analyses were performed using SPSS vers. 10.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA with Duncan’s multiple range test was used to examine the difference between groups. The p-values <0.05 were considered significant if not otherwise stated.

Results

1. GAEE inhibited LPS-PG-stimulated NOS activity and PGE\textsubscript{2} production in HGF-1 cells

Anti-inflammatory activity of GAEE was investigated in LPS-PG-stimulated HGF-1 cells. As shown in Figure 1, LPS-PG induced NOS activity and PGE\textsubscript{2} production were significantly attenuated by GAEE treatment in a dose-dependent manner without any cytotoxicity (data not shown) in HGF-1 cells.

2. GAEE inhibited iNOS and COX-2 expression through the nuclear factor (NF)-κB and activator protein (AP)-1 activations in LPS-PG-stimulated HGF-1 cells

Western blot analysis was conducted in order to analyze the molecular mechanisms underlying the anti-inflammatory activity of GAEE. As shown in Figure 2, protein expression levels of iNOS and COX-2 induced by LPS-PG were also significantly

![Figure 1. Gelidium amansii ethanol extract (GAEE) inhibited nitric oxide (NO) (A) and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) (B) production in P. gingivalis lipopolysaccharide (LPS-PG)-stimulated human gingival fibroblast-1 cells. Data represent the mean±standard deviation of triplicate experiments. Values sharing the same superscript are not significantly different at p<0.05 by Duncan’s multiple range test. Conc.: concentration.](www.ijcpd.org)
attenuated by GAEE treatment in a dose-dependent manner.

NF-κB and AP-1 are the important transcription factors which can regulate the expression of iNOS and COX-2. In order to identify whether GAEE exhibits the anti-inflammatory effect through the regulation of both transcription factors, the phosphorylated status of p65 and c-jun was analyzed by western blot. As shown in Figure 3, phosphorylation of p65 and c-jun was inhibited by GAEE treatment in a dose-dependent manner. Therefore, GAEE treatment ameliorated LPS-PG-induced inflammatory response through NF-κB and AP-1 inhibition in HGF-1 cells.

3. GAEE inhibited ERK phosphorylation in LPS-PG-stimulated HGF-1 cells

Phosphorylated status of mitogen-activated protein kinases (MAPKs) was analyzed to identify the upstream signaling molecules that can regulate NF-κB and AP-1 activations. As shown in Figure 4, GAEE inhibited ERK and p38 phosphorylations while Akt and JNK were not given any effect in HGF-1 cells. These results suggest that inhibition of ERK and p38 phosphorylations by GAEE treatment may contribute to reduce LPS-PG-induced NF-κB and AP-1 activations and consequently reduce NOS activity and PGE2 production in HGF-1 cells.

Discussion

COX-2 has discovered in 1991 and a lot of anti-inflammatory candidates have been tested for COX-2 and PG selective inhibitors of inflammatory lesions [16]. Inflammation is a complex defensive immune mechanism against injuries and endogenous toxins [17]. In this study, anti-inflammatory activity of

Figure 2. Gelidium amansii ethanol extract (GAEE) inhibited protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) in P. gingivalis lipopolysaccharide (LPS-PG)-stimulated human gingival fibroblast cells. Panel A shows protein expression levels of iNOS and COX-2 by GAEE. All signals were normalized to protein levels of actin, an internal control, and expressed as a ratio (panel B). Data represent the mean ± standard deviation of triplicate experiments. Values sharing the same superscript are not significantly different at p < 0.05 by Duncan’s multiple range test. Conc.: concentration.

Figure 3. Gelidium amansii ethanol extract (GAEE) inhibited phosphorylation of transcription factors, nuclear factor-κB and activator protein-1, in P. gingivalis lipopolysaccharide (LPS-PG)-stimulated human gingival fibroblast-1 cells. Panel A shows phosphorylated status of p65 and c-jun by GAEE. All signals were normalized to protein levels of actin, an internal control, and expressed as a ratio (panel B). Data represent the mean ± standard deviation of triplicate experiments. Values sharing the same superscript are not significantly different at p < 0.05 by Duncan’s multiple range test. Conc.: concentration.
Chung-Mu Park and Hyun-Seo Yoon: Anti-Inflammatory Effects of *Gelidium amansii* Ethanol Extract in HGF-1 Cells

**Figure 4.** *Gelidium amansii* ethanol extract (GAEE) inhibited phosphorylation of extracellular regulated kinase (ERK) and p38 in *P. gingivalis* lipopolysaccharide (LPS-PG)-stimulated human gingival fibroblast cells. Panel A shows protein expression levels of p-Akt, p-ERK, p-JNK, and p-p38 by GAEE. All signals were normalized to protein levels of Akt, ERK, JNK, and p38, internal controls, and expressed as a ratio (panel B). Data represent the mean ± standard deviation of triplicate experiments. Values sharing the same superscript are not significantly different at p < 0.05 by Duncan’s multiple range test. Conc.: concentration.

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GAEE as a COX-2 inhibitor in LPS-PG stimulated HGF-1 cells. Moreover, inflammation in periodontium can be initiated by periodontopathic bacteria, *P. gingivalis*, which can destroy the periodontal ligament and alveolar bone [4]. In this study, LPS from *P. gingivalis* has been applied in order to identify the anti-inflammatory activity in HGF-1 cells. As shown in Figure 1 and 2, LPS-PG induced NO and PGE2 production and their corresponding enzymes, iNOS and COX-2, which was significantly ameliorated by GAEE treatment in a dose-dependent manner. A gingival fibroblast is a major cell type constituting human periodontal connective tissue and plays an important role in the development of periodontal disease [18].

Gingival fibroblast can produce various inflammatory mediators, such as iNOS, COX-2, IL-6 and 8 [18]. Among them, excessive production of NO by iNOS, the enzyme that can synthesize NO from l-arginine, is associated with inflammatory diseases and development of cancer [19]. COX-2 is the enzyme that produces PGE2 from arachidonic acid and abnormally up-regulated COX-2 expression is usually found in a lot of premalignant and malignant tissues [20]. The expression of iNOS and COX-2 is regulated by inflammatory transcription factors, including NF-κB and AP-1. NF-κB ubiquitously exists in the cytoplasm, which consists of p50 and p65 subunits bound to an inhibitory protein, IκBα, while AP-1 is composed of homo- or heterodimers with the jun and fos families. In response to LPS stimulation, activation of NF-κB and AP-1 is regulated by phosphorylated p65 and c-jun, each subunit of NF-κB and AP-1, respectively [21]. As shown in Figure 3, GAEE suppressed p65 and c-jun phosphorylation in a dose-dependent manner, which GAEE suppressed iNOS and COX-2 expression through the inhibition of NF-κB and AP-1. These results suggested that GAEE had an anti-inflammatory effect in LPS-PG stimulated HGF-1 cells.

Previous studies have reported that inflammation can be regulated through a series of MAPKs and Akt activation followed by NF-κB and AP-1 phosphorylation [22]. So far, a lot of candidates for anti-inflammatory agents have been evaluated for their inhibitory effects on MAPKs and Akt signaling pathways [23]. In this study, *G. amansii*, one of the seaweeds, was evaluated as an anti-inflammatory agent in HGF-1 cells. As shown in Figure 4, the phosphorylated status of MAPKs and and Akt were measured in order to analyze the regulation of upstream signaling molecules related to NF-κB and AP-1 modulation in LPS-PG-stimulated HGF-1 cells. GAEE inhibited ERK and p38 phosphorylations but did not give any effect on JNK and Akt activations. These results imply that GAEE ameliorates LPS-PG induced inflammation by NF-κB and AP-1 inactivations followed by inhibiting ERK and p38 phosphorylation in HGF-1 cells.

Consequently, this study supports that GAEE significantly inhibits NO and PGE2 production through the NF-κB/AP-1/MAPK signaling cascades in LPS-PG-stimulated HGF-1 cells, which GAEE might be potential candidate therapeutics to treat gingival inflammation.
Conclusion

This study tried to analyze the anti-inflammatory activity of GAEE on LPS-PG stimulated HGF-1 cells. LPS-PG induced inflammatory mediators, iNOS and COX-2 were significantly attenuated by GAEE treatment in a dose-dependent manner. Phosphorylated status of p65 and c-jun, each subunit of NF-κB and AP-1 was also dose-dependently inhibited by GAEE treatment. GAEE inhibited phosphorylation of ERK but did not give any effect on other MAPKs and phosphoinositide 3-kinase/Akt molecules. Consequently, GAEE ameliorates LPS-PG-induced inflammatory mediators by blocking NF-κB, AP-1, and ERK activation in HGF-1 cells.

Acknowledgements

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