学 位 論 文

論文題目

LPS 刺激ヒト歯肉線維芽細胞における Extracellular Signal-Regulated Kinase のリン酸化抑制を介した葛根湯 の炎症反応抑制効果

喜多村洋幸

大学院歯学独立研究科　硬組織疾患制御再建学講座
（主指導教員：荒　敏昭　講師　）

松本歯科大学大学院歯学独立研究科博士(歯学)学位申請論文

Title
Preventive Effects of a Kampo Medicine, Kakkonto, on Inflammatory Responses via the Suppression of Extracellular Signal-Regulated Kinase Phosphorylation in Lipopolysaccharide-Treated Human Gingival Fibroblasts.

Hiroyuki Kitamura

Department of Hard Tissue Research, Graduate School of Oral Medicine
（Chief Academic Advisor：Lecturer Toshiaki Ara ）

The thesis submitted to the Graduate School of Oral Medicine, Matsumoto Dental University, for the degree Ph.D. (in Dentistry)

要旨

【目的】

これまでに学位申請者の所属する研究グループは漢方薬（小柴胡湯、黄連湯、半夏瀉心湯）がヒト歯肉線維芽細胞を LPS 刺激した際に産生されるプロスタグランジン E_2（PGE_2）量を低下させることを報告してきた。本研究では一般的に使用されている葛根湯を選択し、その作用についてヒト歯肉組織由来の細胞培養実験系を用いて検討した。

【対象および方法】

通法に従い埋伏歯抜去時の遊離歯肉片からヒト歯肉線維芽細胞を培養した。歯肉線維芽細胞を *Porphyromonas gingivalis* 由来 LPS（10 ng/ml）で 24 時間刺激した後に培養上清中に産生された PGE_2、IL-6、IL-8 量を ELISA にて測定し、刺激終了時の生細胞数で補正した。これらの実験系に葛根湯（0.01-1mg/ml）を同時に添加することにより葛根湯の作用を検討した。シクロオキシゲナーゼ（COX）活性の阻害能は Cayman 社の COX Inhibitor Screening Assay キットにより測定した。ホスホリパーゼ A_2（PLA_2）および COX-2、アネキシン 1、Extracellular Signal-Regulated Kinase（ERK）、リン酸化 ERK の量はウェスタンブロット法で検討した。

【結果】

葛根湯は 1 mg/ml までは細胞の生存率にほとんど影響を与えなかったが、2 mg/ml 以上の濃度では生存率を低下させたため以降の実験では葛根湯の最大濃度を 1 mg/ml とした。

葛根湯は LPS 刺激により歯肉線維芽細胞から産生される PGE_2 量を濃度依存的に減少させた。一方、葛根湯は LPS 刺激なしの場合に産生される PGE_2 量に影響を与えなかった。また葛根湯は LPS 刺激による IL-6 および IL-8 産生量を増加させた。

葛根湯は COX-1 活性をわずかに低下させたが、COX-2 活性には影響を与えなかった。葛根湯はアラキドン酸カスケードの分子である PLA_2 および COX-2、アネキシン 1（PLA_2 活性を抑制する分子）の産生量に影響を与えなかった。しかし、葛根湯は LPS 刺激による ERK のリン酸化を抑制した。

【考察】

ヒト歯肉線維芽細胞を用いた実験系において、葛根湯が PGE_2 の産生を抑制することから抗炎症作用を示すことが示唆された。これまでに ERK のリン酸化が PLA_2 の活性化を引き起こすことが報告されており、葛根湯は LPS 刺激による ERK のリン酸化を抑制した。したがって、葛根湯が PLA_2 の活性化を抑制し、アラキドン酸の産生量およびそれに引く続く PGE_2 の産生量を低下させたものと考えられえる。

歯周病は歯肉の炎症と歯周組織の破壊を伴う疾患であり、重度の症例では歯槽骨の破壊を引き起こす。ケミカルメディエーターであるプロスタグランジン E$_2$（PGE$_2$）および炎症性サイトカインであるインターロイキン（IL）-6 や IL-8 は炎症反応や組織破壊において重要な役割を果たしている。

　本研究で我々は、漢方薬の一つである葛根湯を使用して、歯周病関連細菌である *Porphyromonas gingivalis* 由来のリポ多糖（LPS）でヒト歯肉線維芽細胞を刺激した際に産生される PGE$_2$ および IL-6、IL-8 量に与える影響を検討した。

　葛根湯は LPS によって産生される PGE$_2$ 量を濃度依存的に低下させたが、LPS 刺激なしの際の PGE$_2$ 産生量に影響を及ぼさなかった。一方、葛根湯は IL-6 と IL-8 の産生量を増加させた。1mg/ml 濃度の葛根湯はシクロオキシゲナーゼ（COX）-1 の活性を約 70%に低下させたが、COX-2 の活性に影響を与えなかった。葛根湯は細胞質ホスホリパーゼ A$_2$（cPLA$_2$）およびアネキシン 1、LPS で誘導された COX-2 の発現量に影響を及ぼさなかった。葛根湯は LPS によって誘導された extracellular signal-regulated kinase（ERK）のリン酸化を抑制した。

　リン酸化型（すなわち活性化型）ERK が cPLA$_2$ をリン酸化することで cPLA$_2$ を活性化させることが報告されている。以上の結果から、葛根湯は ERK のリン酸化を抑制することで cPLA$_2$ のリン酸化およびその活性化を抑制し、その結果 PGE$_2$ の産生量を低下させると考えられた。したがって、葛根湯は歯周病の炎症症状の改善に有効である可能性が考えられる。

Research Article

Preventive Effects of a Kampo Medicine, Kakkonto, on Inflammatory Responses via the Suppression of Extracellular Signal-Regulated Kinase Phosphorylation in Lipopolysaccharide-Treated Human Gingival Fibroblasts

Hiroyuki Kitamura,[1] Hiroko Urano,[2] and Toshiaki Ara[1,3]

[1] *Department of Hard Tissue Research, Graduate School of Oral Medicine, Matsumoto Dental University, Shiojiri, Nagano 399-0781, Japan*

[2] *Institute for Oral Science, Graduate School of Oral Medicine, Matsumoto Dental University, Shiojiri, Nagano 399-0781, Japan*

[3] *Department of Pharmacology, Matsumoto Dental University, 1780 Gobara, Hirooka, Shiojiri, Nagano 399-0781, Japan*

Correspondence should be addressed to Toshiaki Ara; ara_t@po.mdu.ac.jp

Periodontal disease is accompanied by inflammation of the gingiva and destruction of periodontal tissues, leading to alveolar bone loss in severe clinical cases. The chemical mediator prostaglandin E_2 (PGE_2) and cytokines such as interleukin- (IL-)6 and IL-8 have been known to play important roles in inflammatory responses and tissue degradation. In the present study, we investigated the effects of a kampo medicine, kakkonto (TJ-1), on the production of prostaglandin E_2 (PGE_2), IL-6, and IL-8 by human gingival fibroblasts (HGFs) treated with lipopolysaccharide (LPS) from *Porphyromonas gingivalis*. Kakkonto concentration dependently suppressed LPS-induced PGE_2 production but did not alter basal PGE_2 levels. In contrast, kakkonto significantly increased LPS-induced IL-6 and IL-8 production. Kakkonto decreased cyclooxygenase- (COX-)1 activity to approximately 70% at 1 mg/mL but did not affect COX-2 activity. Kakkonto did not affect cytoplasmic phospholipase A_2 ($cPLA_2$), annexin1, or LPS-induced COX-2 expression. Kakkonto suppressed LPS-induced extracellular signal-regulated kinase (ERK) phosphorylation, which is known to lead to ERK activation and $cPLA_2$ phosphorylation. These results suggest that kakkonto decreased PGE_2 production by inhibition of ERK phosphorylation which leads to inhibition of $cPLA_2$ phosphorylation and its activation. Therefore, kakkonto may be useful to improve gingival inflammation in periodontal disease.

1. Introduction

Periodontal disease is accompanied by inflammation of the gingiva and destruction of periodontal tissues, leading to alveolar bone loss in severe clinical cases. Prostaglandin E_2 (PGE_2), interleukin- (IL-)6, and IL-8 are known to play important roles in inflammatory responses and tissue degradation. PGE_2 has several functions in vasodilation, the enhancement of vascular permeability and pain, and the induction of osteoclastogenesis and is believed to play important roles in inflammatory responses and alveolar bone resorption in periodontal disease [1]. IL-6 has the ability

to induce osteoclastogenesis [2, 3]. IL-8 acts as a chemoattractant for neutrophils [4] that produce proteases such as cathepsin, elastase, and matrix metalloproteinase- (MMP-)8, leading to tissue degradation.

Recently, we reported that several kampo medicines, shosaikoto [5], hangeshashinto [6], and orento [7], suppress lipopolysaccharide- (LPS-) induced PGE_2 production by HGFs and suggested that these kampo medicines have anti-inflammatory effects in periodontal disease. Another kampo medicine, kakkonto (TJ-1), has been clinically used for various diseases such as the common cold, coryza, the initial stage of febrile diseases, and inflammatory diseases. There

Japanese name	Latin name	Amount (g)	Amount* (g/g of product)
Kakkon	*Puerariae Radix*	4.0	0.111
Taiso	*Zizyphi Fructus*	3.0	0.083
Mao	*Ephedrae Herba*	3.0	0.083
Kanzo	*Glycyrrhizae Radix*	2.0	0.056
Keihi	*Cinnamomi Cortex*	2.0	0.056
Shakuyaku	*Paeoniae Radix*	2.0	0.056
Shokyo	*Zingiberis Rhizoma*	2.0	0.056
Total		18.0	0.500

*7.5 g of kakkonto product contains 3.75 g of a dried extract of the mixed crude drugs.

are several reports that kakkonto shows antiallergic effects [8, 9] and antiviral effects [10–13] in animal and *in vitro* experimental models. For anti-inflammatory effects, kakkonto has been reported to decrease PGE_2 production in cultured rabbit astrocytes [14]. Therefore, we considered the possibility that kakkonto decreases PGE_2 production by human gingival fibroblasts (HGFs) and has anti-inflammatory effects with respect to periodontal disease. However, the anti-inflammatory effects of kakkonto are not adequately understood.

HGFs are the most prominent cells in periodontal tissue. Moreover, LPS-treated HGFs produce inflammatory chemical mediators such as PGE_2 and inflammatory cytokines such as IL-6 and IL-8 [2, 15, 16]. Moreover, because HGFs have sustained production of PGE_2 [17], IL-6, and IL-8 [18] in the presence of LPS, these mediators and cytokines in periodontal tissues are thought to be derived from HGFs. Therefore, we believe that examining the effects of drugs on HGFs, as well as on monocytes and macrophages, is important in the study of periodontal disease. In the present study, we examined the effect of kakkonto on LPS-induced PGE_2, IL-6, and IL-8 production using this *in vitro* model.

2. Materials and Methods

2.1. Reagents. Kakkonto was purchased from Tsumura & Co. (Tokyo, Japan; lot number: D23122), and its components are listed in Table 1. Kakkonto was suspended in Dulbecco's modified Eagle's medium (D-MEM, Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 mg/mL streptomycin (culture medium) and was rotated at 4°C overnight. Then, the suspension was centrifuged and the supernatant was filtrated through a 0.45 μm-pore membrane. Phorbol 12-myristate 13-acetate (PMA) purchased from Sigma. Other reagents were purchased from Nacalai tesque (Kyoto, Japan). LPS from *Porphyromonas gingivalis* 381 was provided by Professor Nobuhiro Hanada (School of Dental Medicine, Tsurumi University, Japan).

2.2. Cells. HGFs were prepared as described previously [6]. In brief, HGFs were prepared from free gingiva during the extraction of an impacted tooth with the informed consent of the subjects who consulted Matsumoto Dental University Hospital. The free gingival tissues were cut into pieces and seeded onto 24-well plates (AGC Techno Glass Co., Chiba, Japan). HGFs were maintained in culture medium at 37°C in a humidified atmosphere of 5% CO. For passage, HGFs were trypsinized, suspended, and plated into new cultures in a 1 : 3 dilution ratio. HGFs were used between the 10th and 15th passages in the assays. This study was approved by the Ethical Committee of Matsumoto Dental University (number 0063).

2.3. Cell Viability. The numbers of cells were measured using WST-8 (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. In brief, HGFs (10,000 cells/well) were seeded in 96-well plates (AGC Techno Glass Co., Chiba, Japan) and incubated in serum-containing medium at 37°C overnight. Then, the cells were treated with various concentrations of kakkonto (0, 0.5, 1, 2, 5, and 10 mg/mL) in the absence or presence of LPS (10 ng/mL) for 24 h (200 μL each well) in quadruplicate for each sample. Then, the media were removed by aspiration and the cells were treated with 100 μL of mixture of WST-8 with culture medium for 2 h at 37°C in CO incubator. Optical density was measured (measured wavelength at 450 nm and reference wavelength at 655 nm) using an iMark microplate reader (Bio-Rad, Hercules, CA, USA), and the mean background value was subtracted from each value. Data is represented as means ± SD ($n = 4$).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). HGFs (10,000 cells/well) were seeded in 96-well plates and incubated in serum-containing medium at 37°C overnight. Then, the cells were treated with various concentrations of kakkonto (0, 0.01, 0.03, 0.1, 0.3, and 1 mg/mL) in the absence or presence of LPS (10 ng/mL) for 24 h (200 μL each well) in triplicate for each sample. After the culture supernatants were collected, viable cell numbers were measured using WST-8 as described above. The concentrations of PGE_2, IL-6, and IL-8 in the culture supernatants were measured by ELISA according to the manufacturer's instructions (PGE_2, Cayman Chemical, Ann Arbor, MI, USA; IL-6 and IL-8, Biosource International Inc., Camarillo, CA, USA) and were adjusted by the number of viable cells. Data are represented as ng or pg per 10,000 cells (mean ± SD, $n = 3$).

2.5. Cyclooxygenase Activity. The effects of kakkonto on the activities of cyclooxygenase (COX)-1 and COX-2 were analyzed using a COX inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. COX activities were evaluated by the measurement of prostaglandin produced from arachidonic acid by COX-1 or COX-2. These values were normalized to a relative value of 100% for cells without LPS or kakkonto treatments, and are represented as means ± SD ($n = 3$).

2.6. Western Blotting. HGFs were cultured in 60 mm dishes and treated with combinations of LPS and kakkonto for the indicated times. Then, cells were washed twice with Tris-buffered saline, transferred into microcentrifuge tubes,
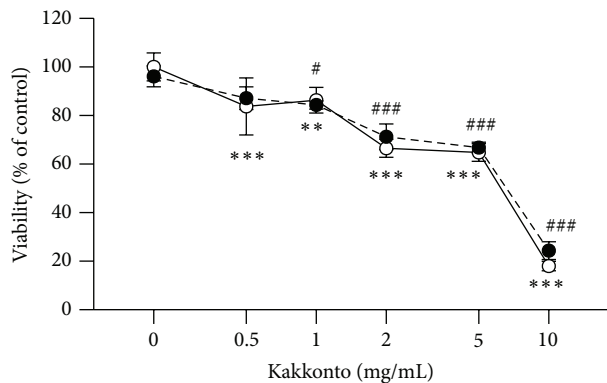
FIGURE 1: Effects of kakkonto on HGFs viability. The effect of kakkonto on the viability of HGFs at 24 h. HGFs were plated in 96-well microplates at 10,000 cells/mL, and media containing LPS and kakkonto were added. Cell numbers were evaluated by WST-8 at 24 h. The optical density (OD) was normalized to a relative value of 100% for cells without LPS or kakkonto treatments and is represented as means ± SD (n = 4). Open circles, treatment without LPS; closed circles, treatment with 10 ng/mL of LPS. $^{**}P < 0.01$ and $^{***}P < 0.001$ (without kakkonto versus with kakkonto in the absence of LPS). $^{#}P < 0.01$ and $^{##}P < 0.001$ (without kakkonto versus with kakkonto in the presence of LPS). P values were calculated by pairwise comparisons and corrected with the Holm method (16 null hypotheses).

and centrifuged at 6,000 ×g for 5 min at 4°C. Supernatants were aspirated and cells were lysed on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethyleneglycol bis(2-aminoethylether) tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 5 μg/mL leupeptin, and 1 μg/mL pepstatin) for 30 min at 4°C. Then, samples were centrifuged at 12,000 ×g for 15 min at 4°C, and supernatants were collected. The protein concentration was measured using a BCA Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL, USA).

The samples (10 μg of protein) were fractionated in a polyacrylamide gel under reducing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare, Uppsala, Sweden). The membranes were blocked with 5% ovalbumin for 1 h at room temperature and incubated with primary antibody for an additional 1 h. The membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized with an ECL Kit (GE Healthcare).

Antibodies against COX-2 (sc-1745, 1 : 500 dilution), cytoplasmic phospholipase A$_2$ (cPLA$_2$, sc-438, 1 : 200 dilution), annexin1 (sc-11387, 1 : 1,000 dilution), and actin (sc-1616, 1 : 1,000 dilution), which detects a broad range of actin isoforms, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against extracellular signal-regulated kinase (ERK; p44/42 MAP kinase antibody, 1 : 1,000 dilution) and phosphorylated ERK (Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody, 1 : 2,000 dilution) were from Cell Signaling Technology (Danvers,

MA, USA). Horseradish peroxidase-conjugated anti-goat IgG (sc-2020, 1 : 20,000 dilution) was from Santa Cruz, and anti-rabbit IgG (1 : 20,000 dilution) and anti-mouse IgG (1 : 20,000 dilution) were from DakoCytomation (Glostrup, Denmark).

2.7. Statistical Analysis. Differences between groups were evaluated by the two-tailed pairwise comparison test with a pooled variance, followed by correction with the Holm method (total 16 null hypotheses; 5 null hypotheses without kakkonto versus with kakkonto in the absence of LPS, 5 null hypotheses without kakkonto versus with kakkonto in the presence of LPS, and 6 null hypotheses without LPS versus with LPS) (Figures 1 and 2). Differences between the control group and experimental groups were evaluated by a two-tailed Dunnett's test (Figure 3).

All computations were performed with the statistical program R (http://www.r-project.org/). Dunnett's test was performed using the "glht" function in the "multcomp" package. Values with $P < 0.05$ were considered significantly different.

3. Results

3.1. Effects of Kakkonto on HGFs Viability. First, we examined the effect of kakkonto on HGFs viability. The viability of HGFs was approximately 90% at up to 1 mg/mL of kakkonto for a 24 h treatment in the absence or presence of LPS (Figure 1). The viabilities were approximately 70% and 20% at 5 mg/mL and 10 mg/mL of kakkonto, respectively (Figure 1). Therefore, we used kakkonto at the concentrations of up to 1 mg/mL in further experiments.

3.2. Effects of Kakkonto on PGE$_2$, IL-6, and IL-8 Production. We examined whether kakkonto affects the production of PGE$_2$ and inflammatory cytokines (IL-6 and IL-8) by HGFs. Because kakkonto affects cell viability, the concentrations of PGE$_2$, IL-6, and IL-8 needed to be adjusted according to viable cell number.

When HGFs were treated with 10 ng/mL of LPS, HGFs produced large amounts of PGE$_2$, IL-6, and IL-8. Indomethacin decreased LPS-induced PGE$_2$ production in a concentration-dependent manner but slightly decreased LPS-induced IL-6 and IL-8 production (data not shown). Kakkonto significantly decreased PGE$_2$ production in a concentration-dependent manner (Figure 2(a)). In the absence of LPS, kakkonto had no effect on PGE$_2$ production (Figure 2(a)). In contrast, kakkonto increased LPS-induced IL-6 and IL-8 production (Figures 2(b) and 2(c)). In the absence of LPS, up to 0.1 mg/mL of kakkonto did not affect IL-6 and IL-8 production, but above 0.3 mg/mL of kakkonto, their concentrations were increased (Figures 2(b) and 2(c)). Similar results were obtained using human skin fibroblast TIG-103 cells (data not shown).

3.3. Effects of Kakkonto on COX Activities. Because PGE$_2$ production is regulated by COX enzymes and suppressed by acid NSAIDs such as aspirin and diclofenac sodium, which inhibit COX activities, we examined whether kakkonto inhibits COX-1 and COX-2 activities. Kakkonto decreased
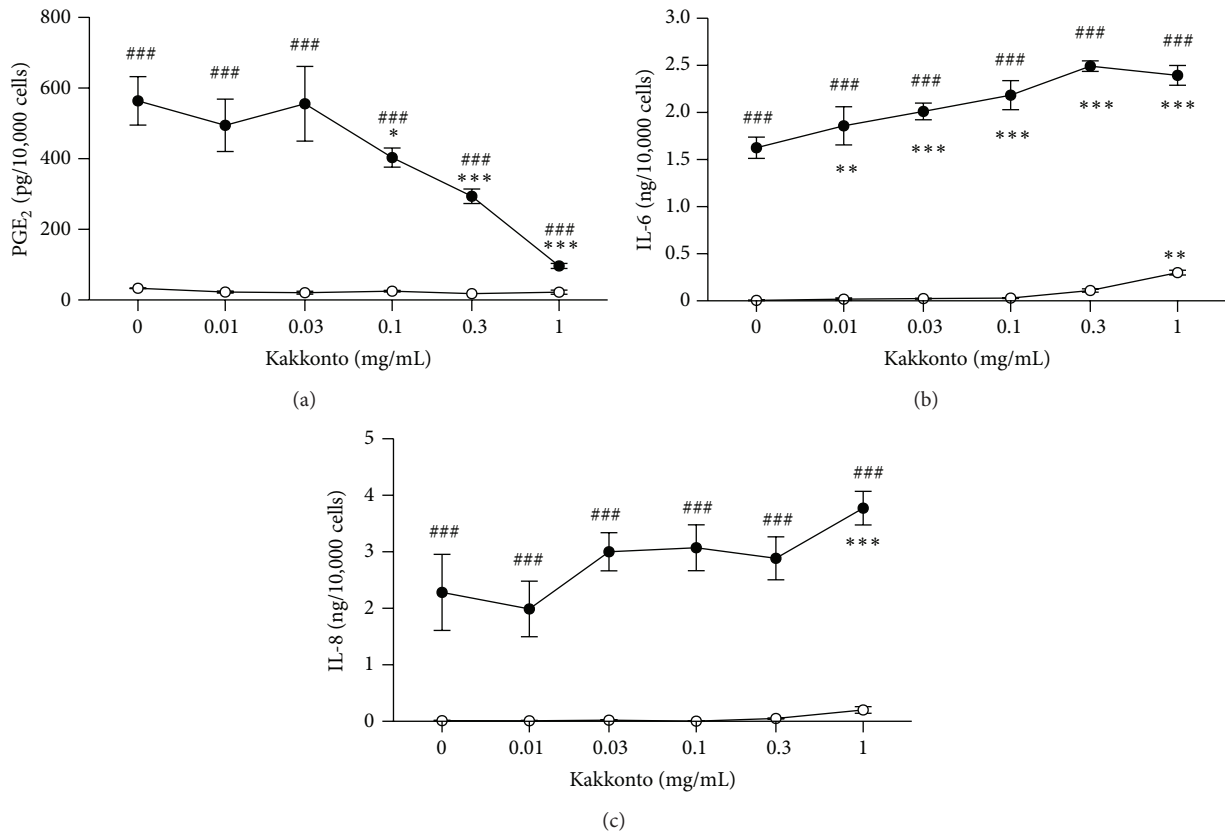
(a)

(b)

(c)

FIGURE 2: Effects of kakkonto on the production of PGE_2, IL-6, and IL-8. HGFs were treated with combinations of LPS (0 and 10 ng/mL) and kakkonto (0, 0.01, 0.3, 0.1, 0.3, and 1 mg/mL) for 24 h. Concentrations of PGE_2 (a), IL-6 (b), and IL-8 (c) were measured by ELISA, adjusted by cell number, and expressed as per 10,000 cells (mean ± SD, $n = 3$). Open circles, treatment without LPS; closed circles, treatment with 10 ng/mL of LPS. **$P < 0.01$ and ***$P < 0.001$ (without kakkonto versus with kakkonto). ##$P < 0.001$ (without LPS versus with LPS). P values were calculated by pairwise comparisons and corrected with the Holm method (16 null hypotheses).
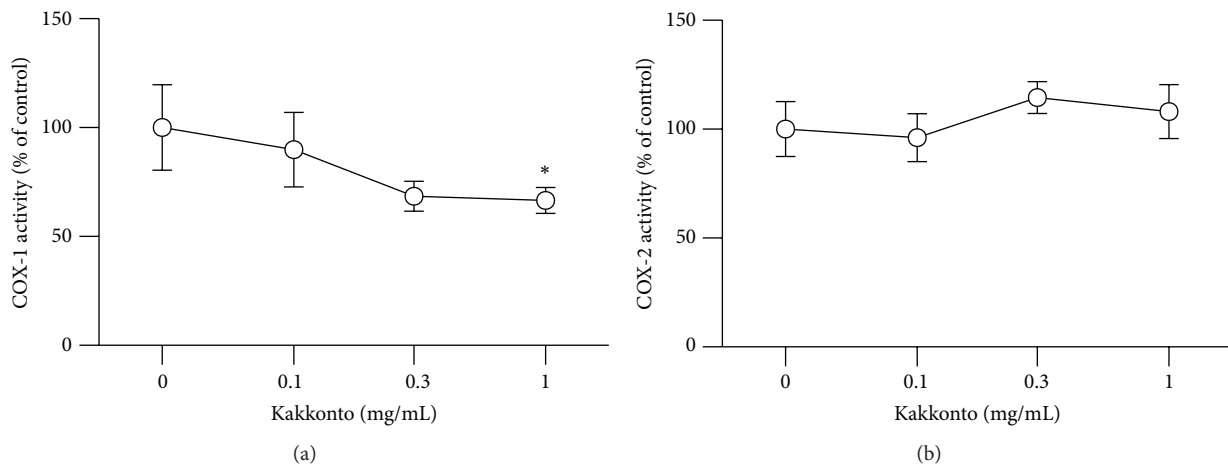

(a)

(b)

FIGURE 3: Effects of kakkonto on COX activities. COX activities were evaluated by measurement of prostaglandin produced from arachidonic acid by COX-1 or COX-2. These values were normalized to a relative value of 100% for cells without LPS or kakkonto treatments and are represented as means ± SD ($n = 3$). *$P < 0.05$ (Dunnett's test).
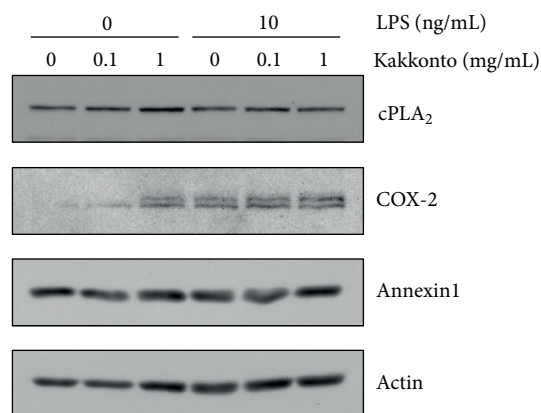
FIGURE 4: Effects of kakkonto on cPLA$_2$, COX-2, and annexin1 expressions. HGFs were treated with a combination of LPS (0 or 10 ng/mL) and kakkonto (0, 0.01, or 1 mg/mL) for 8 h, and protein levels were examined by western blotting.
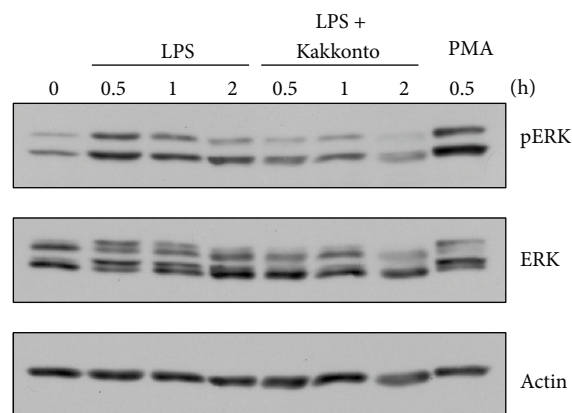

FIGURE 5: Effects of kakkonto on LPS-induced ERK phosphorylation. HGFs were untreated (0 h), treated with LPS (10 ng/mL), or treated with both LPS and kakkonto (1 mg/mL) for 0.5, 1, and 2 h. As a positive control, HGFs were treated with 1 μM of PMA for 0.5 h. Western blotting was performed using antiphosphorylated ERK or anti-ERK antibodies. pERK: phosphorylated ERK. Upper band indicates ERK1 (p44 MAPK) and lower band indicates ERK2 (p42 MAPK).

COX-1 activity to approximately 70% at 1 mg/mL but did not affect COX-2 activity (Figure 3).

3.4. Effects of Kakkonto on Molecular Expression in the Arachidonic Acid Cascade.

We examined whether kakkonto affects the expression of molecules in the arachidonic acid cascade. cPLA$_2$ is the most upstream enzyme in the arachidonic acid cascade and releases arachidonic acid from plasma membranes. Kakkonto did not alter cPLA$_2$ expression in the absence or presence of LPS (Figure 4). COX-2 was not detected in the absence of LPS. Treatment with kakkonto alone increased COX-2 expression. However, kakkonto did not alter LPS-induced COX-2 expression (Figure 4). Annexin1, also named lipocortin1, is an anti-inflammatory mediator produced by glucocorticoids that inhibit cPLA$_2$ activity [19, 20]. However, neither LPS nor kakkonto showed an effect on annexin1 expression (Figure 4).

3.5. Effects of Kakkonto on ERK Phosphorylation.

cPLA$_2$ is reported to be directly phosphorylated at Ser505 by ERK, resulting in cPLA$_2$ activation [21, 22]. Therefore, we examined whether kakkonto suppresses LPS-induced ERK phosphorylation. ERK phosphorylation was enhanced at 0.5 h after LPS treatment and thereafter was attenuated. One mg/mL of kakkonto suppressed LPS-induced ERK phosphorylation at 0.5 h to 2 h (Figure 5).

4. Discussion

In the present study, we examined the effect of kakkonto on LPS-induced PGE$_2$, IL-6, and IL-8 production by HGFs. Kakkonto concentration dependently decreased LPS-induced PGE$_2$ production but did not affect PGE$_2$ production without LPS treatment, similar to shosaikoto, hange-shashinto, and orento [5–7]. Moreover, kakkonto suppressed LPS-induced ERK phosphorylation. In contrast, kakkonto increased LPS-induced IL-6 and IL-8 production. It is widely known that PGE$_2$ leads to inflammatory responses such

as vasodilation, enhanced vascular permeability, and pain generation [1]. Acid non-steroidal anti-inflammatory drugs NSAIDs show anti-inflammatory effects by suppression of PGE$_2$ production, even though they do not affect IL-6 and IL-8 production. Our findings showing that kakkonto decreases LPS-induced PGE$_2$ production suggest that kakkonto also has anti-inflammatory effects in periodontal disease and that its effects are mainly mediated by suppression of PGE$_2$ production even though kakkonto increased LPS-induced IL-6 and IL-8 production.

Our results showed that kakkonto suppressed LPS-induced ERK phosphorylation in HGFs. Previously, we demonstrated that orento inhibits LPS-induced ERK phosphorylation and cPLA$_2$ activation, leading to the suppression of PGE$_2$ production in HGFs [7]. Therefore, we consider that kakkonto decreased LPS-induced PGE$_2$ production through the suppression of ERK phosphorylation in HGFs.

Although kakkonto increased COX-2 expression in the absence of LPS, kakkonto did not alter PGE$_2$ production. We consider a likely reason to be the suppression of cPLA$_2$ activation through the inhibition of ERK phosphorylation and/or the suppression of COX-1 activity. However, the components that induce COX-2 expression remain unknown.

Our results showed that kakkonto increased LPS-induced IL-6 and IL-8 production by HGFs. Previously, we reported that the activation of the protein kinase A (PKA) pathway by adrenaline or aminophylline increases LPS-induced IL-6 and IL-8 production in HGFs [23] and that H-89, a PKA inhibitor, decreases LPS-induced IL-6 and IL-8 production [23, 24]. Therefore, kakkonto may activate the PKA pathway.

In general, steroidal anti-inflammatory drugs (SAIDs) suppress the expression of cPLA$_2$, COX-2, and inflammatory cytokines (such as IL-6 and IL-8) and induce the expression of annexin1. However, kakkonto did not affect cPLA$_2$, annexin1, or LPS-induced COX-2 expression, and it increased IL-6 and

IL-8 production. This therefore suggests that the mechanism by which kakkonto decreases PGE_2 production is different from that of SAIDs.

Many studies have demonstrated that NSAID administration prevents gingival inflammation [25] and several clinical studies have indicated that the concentration of PGE_2 in gingival crevicular fluid (GCF) is increased in periodontal disease [26] and is decreased by oral administration or mouthwash with NSAIDs [27, 28]. Considering that both NSAIDs and kakkonto suppress PGE_2 production, it is possible that administration of kakkonto also decreases the PGE_2 concentration in GCF and results in the improvement of gingival inflammation. Therefore, kakkonto may be useful for the improvement of gingival inflammation in periodontal disease. Importantly, kakkonto did not affect the basal level of PGE_2, although kakkonto decreased COX-1 activity to approximately 70%. Because PGE_2 produced by COX-1 protects gastric mucosa, these results suggest that kakkonto may cause minimal gastrointestinal dysfunction.

5. Conclusion

We demonstrated that kakkonto suppresses LPS-induced ERK phosphorylation, resulting in the suppression $cPLA_2$ activation and further PGE_2 production by HGFs. These results suggest that kakkonto is clinically useful for the improvement of inflammatory responses in periodontal disease.

Ethical Approval

This study was approved by the Ethical Committee of Matsumoto Dental University (no. 0063).

Conflict of Interests

The authors have no conflict of interests to disclose.

Acknowledgments

References

[1] K. Noguchi and I. Ishikawa, "The roles of cyclooxygenase-2 and prostaglandin E_2 in periodontal disease," *Periodontology 2000*, vol. 43, no. 1, pp. 85–101, 2007.

[2] P. M. Bartold and D. R. Haynes, "Interleukin-6 production by human gingival fibroblasts," *Journal of Periodontal Research*, vol. 26, no. 4, pp. 339–345, 1991.

[3] H. Takada, J. Mihara, I. Morisaki, and S. Hamada, "Induction of interleukin-1 and -6 in human gingival fibroblast cultures stimulated with *Bacteroides* lipopolysaccharides," *Infection and Immunity*, vol. 59, no. 1, pp. 295–301, 1991.

[4] H. Okada and S. Murakami, "Cytokine expression in periodontal health and disease," *Critical Reviews in Oral Biology and Medicine*, vol. 9, no. 3, pp. 248–266, 1998.

[5] T. Ara, Y. Maeda, Y. Fujinami, Y. Imamura, T. Hattori, and P. L. Wang, "Preventive effects of a Kampo medicine, Shosaikoto, on inflammatory responses in LPS-treated human gingival fibroblasts," *Biological and Pharmaceutical Bulletin*, vol. 31, no. 6, pp. 1141–1144, 2008.

[6] Y. Nakazono, T. Ara, Y. Fujinami, T. Hattori, and P. L. Wang, "Preventive effects of a kampo medicine, hangeshashinto on inflammatory responses in lipopolysaccharide-treated human gingival fibroblasts," *Journal of Hard Tissue Biology*, vol. 19, no. 1, pp. 43–50, 2010.

[7] T. Ara, K. Honjo, Y. Fujinami, T. Hattori, Y. Imamura, and P. L. Wang, "Preventive effects of a kampo medicine, orento on inflammatory responses in lipopolysaccharide treated human gingival fibroblasts," *Biological and Pharmaceutical Bulletin*, vol. 33, no. 4, pp. 611–616, 2010.

[8] Y. Ozaki, "Studies on antiinflammatory effect of Japanese oriental medicines (Kampo medicines) used to treat inflammatory diseases," *Biological and Pharmaceutical Bulletin*, vol. 18, no. 4, pp. 559–562, 1995.

[9] T. Yamamoto, K. Fujiwara, M. Yoshida et al., "Therapeutic effect of kakkonto in a mouse model of food allergy with gastrointestinal symptoms," *International Archives of Allergy and Immunology*, vol. 148, no. 3, pp. 175–185, 2009.

[10] K. Nagasaka, M. Kurokawa, M. Imakita, K. Terasawa, and K. Shiraki, "Efficacy of Kakkon-to, a traditional herb medicine, in herpes simplex virus type 1 infection in mice," *Journal of Medical Virology*, vol. 46, no. 1, pp. 28–34, 1995.

[11] M. Kurokawa, M. Tsurita, J. Brown, Y. Fukuda, and K. Shiraki, "Effect of interleukin-12 level augmented by Kakkon-to, a herbal medicine, on the early stage of influenza infection in mice," *Antiviral Research*, vol. 56, no. 2, pp. 183–188, 2002.

[12] M. S. Wu, H. R. Yen, C. W. Chang et al., "Mechanism of action of the suppression of influenza virus replication by Ko-Ken Tang through inhibition of the phosphatidylinositol 3-kinase/Akt signaling pathway and viral RNP nuclear export," *Journal of Ethnopharmacology*, vol. 134, no. 3, pp. 614–623, 2011.

[13] J. S. Chang, K. C. Wang, D. E. Shieh, F. F. Hsu, and L. C. Chiang, "Ge-Gen-Tang has anti-viral activity against human respiratory syncytial virus in human respiratory tract cell lines," *Journal of Ethnopharmacology*, vol. 139, no. 1, pp. 305–310, 2012.

[14] M. Kutsuwa, N. Nakahata, M. Kubo, K. Hayashi, and Y. Ohizumi, "A comparative study of Kakkon-to and Keishi-to on prostaglandin E_2 release from rabbit astrocytes," *Phytomedicine*, vol. 5, no. 4, pp. 275–282, 1998.

[15] H. J. Sismey-Durrant and R. M. Hopps, "Effect of lipopolysaccharide from *Porphyromonas gingivalis* on prostaglandin E_2 and interleukin-1-β release from rat periosteal and human gingival fibroblasts *in vitro*," *Oral Microbiology and Immunology*, vol. 6, no. 6, pp. 378–380, 1991.

[16] M. Tamura, M. Tokuda, S. Nagaoka, and H. Takada, "Lipopolysaccharides of *Bacteroides intermedius* (*Prevotella intermedia*) and *Bacteroides* (*Porphyromonas*) gingivalis induce interleukin-8 gene expression in human gingival fibroblast cultures," *Infection and Immunity*, vol. 60, no. 11, pp. 4932–4937, 1992.

[17] T. Ara, Y. Fujinami, Y. Imamura, and P. L. Wang, "Lipopolysaccharide-treated human gingival fibroblasts continuously produce PGE_2," *Journal of Hard Tissue Biology*, vol. 17, no. 3, pp. 121–124, 2008.

[18] T. Ara, K. Kurata, K. Hirai et al., "Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease," *Journal of Periodontal Research*, vol. 44, no. 1, pp. 21–27, 2009.

[19] C. Gupta, M. Katsumata, A. S. Goldman, R. Herold, and R. Piddington, "Glucocorticoid-induced phospholipase A_2-inhibitory proteins mediate glucocorticoid teratogenicity *in vitro*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 4 I, pp. 1140–1143, 1984.

[20] B. P. Wallner, R. J. Mattaliano, C. Hession et al., "Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity," *Nature*, vol. 320, no. 6057, pp. 77–81, 1986.

[21] L. L. Lin, M. Wartmann, A. Y. Lin, J. L. Knopf, A. Seth, and R. J. Davis, "cPLA$_2$ is phosphorylated and activated by MAP kinase," *Cell*, vol. 72, no. 2, pp. 269–278, 1993.

[22] M. A. Gijón, D. M. Spencer, A. L. Kaiser, and C. C. Leslie, "Role of phosphorylation sites and the C2 domain in regulation of cytosolic phospholipase A_2," *Journal of Cell Biology*, vol. 145, no. 6, pp. 1219–1232, 1999.

[23] T. Ara, Y. Fujinami, H. Urano, K. Hirai, T. Hattori, and H. Miyazawa, "Protein kinase A enhances lipopolysaccharide-induced IL-6, IL-8, and PGE$_2$ production by human gingival fibroblasts," *Journal of Negative Results in BioMedicine*, vol. 11, article 10, 2012.

[24] A. Kamemoto, T. Ara, T. Hattori, Y. Fujinami, Y. Imamura, and P. L. Wang, "Macrolide antibiotics like azithromycin increase lipopolysaccharide-induced IL-8 production by human gingival fibroblasts," *European Journal of Medical Research*, vol. 14, no. 7, pp. 309–314, 2009.

[25] G. E. Salvi and N. P. Lang, "Host response modulation in the management of periodontal diseases," *Journal of Clinical Periodontology*, vol. 32, no. 6, pp. 108–129, 2005.

[26] S. Offenbacher, D. H. Farr, and J. M. Goodson, "Measurement of prostaglandin E in crevicular fluid," *Journal of Clinical Periodontology*, vol. 8, no. 4, pp. 359–367, 1981.

[27] M. M. Abramson, L. F. Wolff, S. Offenbacher, D. M. Aeppli, N. D. Hardie, and H. M. Friedman, "Flurbiprofen effect on gingival crevicular fluid prostaglandin and thromboxane levels in humans," *Journal of Periodontal Research*, vol. 27, no. 5, pp. 539–543, 1992.

[28] M. K. Jeffcoat, M. S. Reddy, S. Haigh et al., "A comparison of topical ketorolac, systemic flurbiprofen, and placebo for the inhibition of bone loss in adult periodontitis," *Journal of Periodontology*, vol. 66, no. 5, pp. 329–338, 1995.