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Original

Preventive Effects of a Kampo Medicine, Hangeshashinto on Inflammatory Responses in Lipopolysaccharide-Treated Human Gingival Fibroblasts

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Abstract：In the present study, we investigated the effects of a Kampo medicine hangeshashinto (TJ-14) on the production of prostaglandin E_2 (PGE_2), interleukin (IL)-6 and IL-8 by human gingival fibroblasts (HGFs) treated with lipopolysaccharide (LPS) from *Porphyromonas gingivalis*. HGFs proliferation was dose-dependently decreased with hangeshashinto at days 3 and 7. However, treatment with LPS (10 ng/ml), hangeshashinto (up to 1 mg/ml) and their combinations for 24 h did not affect the viability of HGFs. Hangeshashinto dose-dependently suppressed LPS-induced PGE_2 production but did not alter basal PGE_2 level. Hangeshashinto weakly decreased LPS-induced IL-6 and IL-8 productions. Hangeshashinto decreased cyclooxygenase (COX)-1 and COX-2 activities to approximately 60% at 1 mg/ml. Hangeshashinto decreased cytoplasmic phospholipase A_2 ($cPLA_2$) expression and LPS-induced COX-2 expression but not affected annexin1 expression. Hangeshashinto weakly 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 hangeshashinto decreased PGE_2 production by (1) suppression of $cPLA_2$ and LPS-induced COX-2 expression, and to a lesser extent, (2) inhibition of COX-2 activity and (3) inhibition of $cPLA_2$ phosphorylation and its activation via inhibition of ERK phosphorylation. Moreover, it is also suggested that hangeshashinto may be useful to improve gingival inflammation in periodontal disease.

Key words: Anti-inflammatory effect, Hangeshashinto, Human gingival fibroblast, Prostaglandin E_2

Introduction

Caries and periodontal disease are two major oral diseases and are considered to be biofilm infectious diseases[1]. In particular, periodontal disease is highly prevalent and can affect most of the world population. Periodontal disease is accompanied by inflammation of the gingiva and destruction of periodontal tissues, leading to alveolar bone loss in severe clinical cases. In severe case which the inflammation is intense after the initial preparation, non-steroidal anti-inflammatory drugs (NSAIDs) were administrated to improve gingival inflammation. In fact, many studies demonstrated that systemic administration of acid NSAIDs prevents gingival inflammation and alveolar bone resorption in animals and humans (reviewed in ref. 2). However, acid NSAIDs are reported to have side effects such as gastrointestinal dysfunction.

Recently, we reported that a kampo medicine shosaikoto (TJ-9) and orento (TJ-120) suppressed lipopolysaccharide (LPS)-induced PGE_2 production by HGFs, and suggested that these kampo medicines have anti-inflammatory effects in periodontal disease[3,4]. Other kampo medicine, hangeshashinto (TJ-14), has been used for inflammatory diseases such as acute or chronic gastrointestinal catarrh, nervous gastritis and stomatitis[5] similar to orento[6]. Hangeshashinto is reported to have the capabilities for the protection of the gastric mucosa[7], the antidiarrheal effect[8,9], the anti-inflammatory effect[9-11] and the enhancement of large intestinal water absorption[12]. From these indications, we considered that hangeshashinto could be useful for periodontal disease.

Human gingival fibroblasts (HGFs) are the most prominent cells in periodontal tissue. And HGFs produce inflammatory chemical mediators such as prostaglandin E_2 (PGE_2) and inflammatory cytokines such as interleukin (IL)-6 and IL-8 when HGFs were treated with LPS[13-16]. Therefore, we regard this experimental system, in which HGFs were treated with LPS, as in vitro periodontal disease model. Moreover, because HGFs sustain to produce PGE_2[17] and IL-6 and IL-8[18] in the presence of LPS, these mediators and cytokines in periodontal tissues are thought to be

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derived from HGFs. Therefore, we consider that the examinations of effect on HGFs, as well as monocytes and macrophages, are important in the study on periodontal disease. In this study, we examined the effect of hangeshashinto on LPS-induced PGE_2, IL-6 and IL-8 productions using this *in vitro* model.

Materials and Methods

Reagents

Powder of hangeshashinto was obtained from Tsumura & Co. (Tokyo, Japan), and its component was indicated in Table 1. Hangeshashinto was suspended in Dulbecco's modified Eagle's medium (D-MEM, Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin (culture medium) and was rotated at 4℃ overnight. Then, the suspension was centrifuged and the supernatant was filtrated through 0.45 μm-pore membrane. LPS from *Porphyromonas gingivalis* 381 was provided by Drs. Tatsuji Nishihara and Nobuhiro Hanada (National Institutes of Public Health, Wako, Japan). Phorbol 12-myristate 13-acetate (PMA, Sigma) was dissolved in dimethyl sulfoxide (DMSO, Nacalai tesque, Kyoto, Japan).

Cells

HGFs were prepared from free gingiva during the extraction of an impacted tooth with the informed consents of the subjects who consulted Matsumoto Dental University Hospital. The free gingival tissues were cut into pieces and seeded on a 24-well plates (AGC Techno Glass Co., Chiba, Japan). HGFs were maintained in Dulbecco's modified Eagle's medium (D-MEM; Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin and 100μg/ml streptomycin, at 37 ℃ in a humidified atmosphere of 5% CO_2. HGFs were used between 10th to 20th passages in the assays. This study was approved by the Ethical Committee of Matsumoto Dental University.

Cell viability

The numbers of cells were measured using WST-8 (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufactures' instructions. In brief, 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℃ in CO_2 incubator. Optical density were measured (measure wavelength at 450 nm and reference wavelength at 655 nm) using a microplate reader (Model 550; Bio-Rad, Hercules, CA). The background value was subtracted from each value, and represented as mean ±standard deviation (S.D.) (n=4).

Cytokine measurement by enzyme-linked immunosorbent assay (ELISA)

HGFs (10,000 cells/well) were seeded in 96-well plates (AGC Techno Glass Co.) and incubated in serum-containing medium at 37℃ overnight. Then, the cells were treated with various concentrations of hangeshashinto (0, 0.01, 0.1 and 1 mg/ml) in the absence or presence of LPS (10 ng/ml) for 24 h (200μl per each well). After the culture supernatants were collected, the viable cell numbers were measured using WST-8. The concentrations of PGE_2, IL-6 and IL-8 in the culture supernatants were measured by ELISA according to the manufactures' instructions (PGE_2, Cayman Chemical, Ann Anbor, MI; IL-6 and IL-8, Biosource International Inc., Camarillo, CA), adjusted by the viable cell numbers, and represented as per 10,000 cells (mean ±S.D, n=3).

Measurement of cyclooxygenase activity

The effects of hangeshashinto on the activities of cyclooxygenase (COX)-1 and COX-2 were analyzed using COX inhibitor screening assay kit (Cayman Chemical, Ann Anbor, MI) according to the manufacture's instructions. COXs activities were evaluated by the measurement of prostaglandin produced from arachidonic acid by COX-1 or COX-2 in triplicate. These values were normalized to a relative value of 100% for the cells without both LPS and hangeshashinto treatment, and represented as mean ± S.D. (n=3).

Western blotting

HGFs were cultured in 60 mm dish and treated with the combinations of LPS and hangeshashinto for indicated time. Then, cells were washed twice with Tris-buffered saline, transferred into microcentrifuge tubes, and centrifuged at 6,000 × g for 5 min at 4℃. Supernatants were aspirated and cell 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, 10μmM sodium fluoride, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5μg/ml leupeptin and 1μg/ml pepstatin) for 30 min at 4℃. Then, samples were centrifuged at 12,000 μg for 15 min at 4℃ and supernatants were collected. The protein concentration was measured using BCA Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL).

The samples (10 μg proteins) were fractionated in polyacrylamide gel under reducing conditions and transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare, Uppsala, Sweden). The membranes were blocked with 5% ovalbumin (Nacalai tesque, Kyoto, Japan) for 1 h at room temperature and incubated with primary antibody for 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 ECL kit (GE Healthcare).

Table 1 The ingredient of hangeshashinto formula

Japanese name	Latin name	amount [g (percent)]
hange	*Pinelliae tuber*	5.0 (27.0)
ogon	*Scutellariae radix*	2.5 (13.5)
kankyo	*Zingiberis siccatum*	2.5 (13.5)
kanzo	*Glycyrrhizae radix*	2.5 (13.5)
taiso	*Zizyphi fructus*	2.5 (13.5)
ninjin	*Ginseng radix*	2.5 (13.5)
oren	*Coptidis rhizoma*	1.0 (5.4)
	total	

Table 2 Effect of hangeshashinto on COX activities

concentration (mg/ml)	COX activity (%)	
	COX-1	COX-2
0	100.0 ± 2.5	100.0 ± 0.9
0.1	105.6 ± 11.8	78.6 ± 13.6
1	66.9 ± 3.5**	59.0 ± 6.0**

Data represent the mean ± S.D. (n=3). $^{**}P < 0.01$, significant differences from control (0 mg/ml) by Dunnett's method.

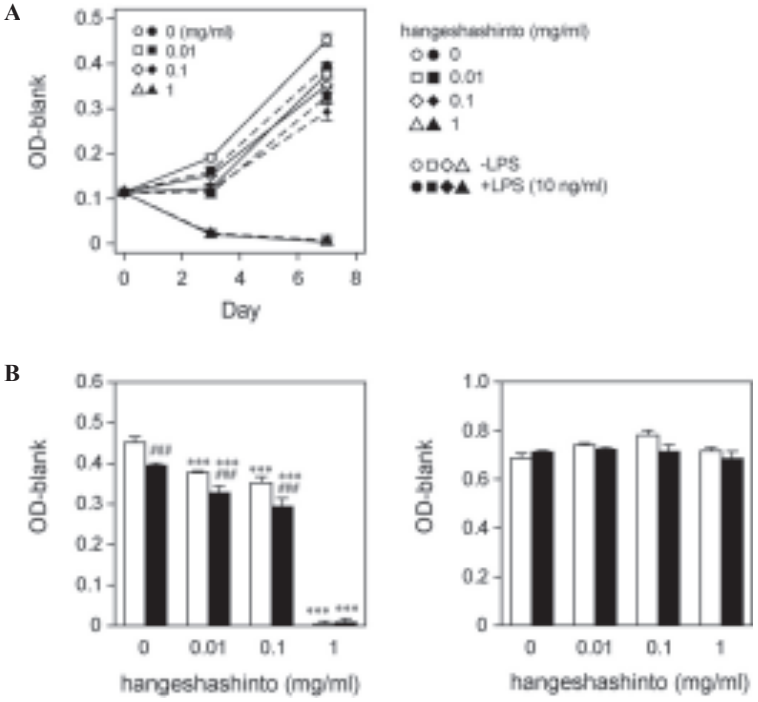

Figure 1. Effect of hangeshashinto on HGFs proliferation. (A, B) HGFs were plated in 96-well microplate at 2,000 cells/ml and were added the media containing LPS (0 and 10 ng/ml) and hangeshashinto (0, 0.01, 0.1 and 1 mg/ml). Cell numbers were evaluated by WST-8 at day 0, 3 and 7 days (A) and at Day 7 (B). (C) The effect of hangeshashinto on viability of HGFs at 24 h. HGFs were plated in 96-well microplate at 10,000 cells/ml and were added the media containing LPS and hangeshashinto. Cell numbers were evaluated by WST-8 at 24 h. The optical density (OD) was expressed as mean ± S.D. (n=4). Open bars, treatment without LPS; closed bars, treatment with 10 ng/ml of LPS. $^{***}P < 0.001$ (without hangeshashinto vs. with hangeshashinto). $^{\#\#}P < 0.001$ (without LPS vs. with LPS). *P* values were calculated by pairwise comparison and corrected with Holm method (10 null hypotheses).

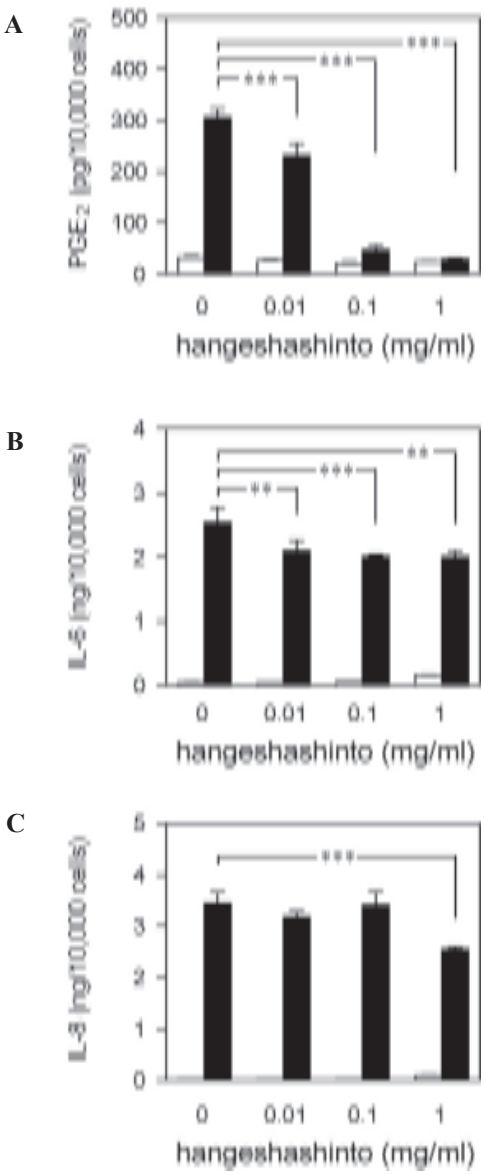

Figure. 2. Effects of hangeshashinto on the production of PGE_2, IL-6 and IL-8. HGFs were treated with the combinations with LPS (0 and 10 ng/ml) and hangeshashinto (0, 0.01, 0.1 and 1 mg/ml) for 24 h, and the concentrations of PGE_2 (A), IL-6 (B) and IL-8 (C) and were measured by ELISA. The concentrations were adjusted by the cell numbers and expressed as per 10,000 cells (mean ± S.D., n=3). Open bars, treatment without LPS; closed bars, treatment with 10 ng/ml of LPS. $^{**}P < 0.01$, $^{***}P < 0.001$. *P* values were calculated by pairwise comparison and corrected with Holm method (10 null hypotheses).

The antibodies against COX-2 (sc-1745, 1:500 dilution), cytoplasmic phospholipase A_2 ($cPLA_2$, sc-438, 1:200 dilution), annexin1 (sc-11387, 1:500 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) and the antibodies against extracellular signal-regulated kinase (ERK; p44/42 MAP kinase antibody, 1:500 dilution) and phosphorylated

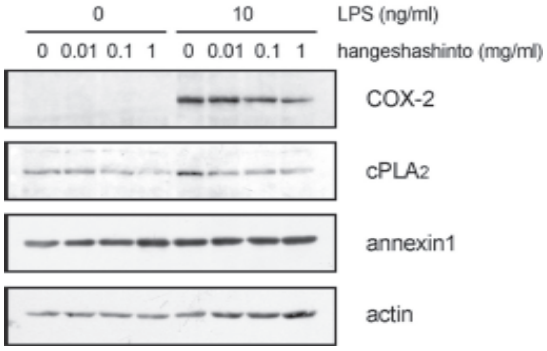

Figure 3. Effects of hangeshashinto on $cPLA_2$, COX-2 and annexin1 expressions. HGFs were treated with the combination of LPS and hangeshashinto for 8 h, and protein levels were examined by Western blotting.

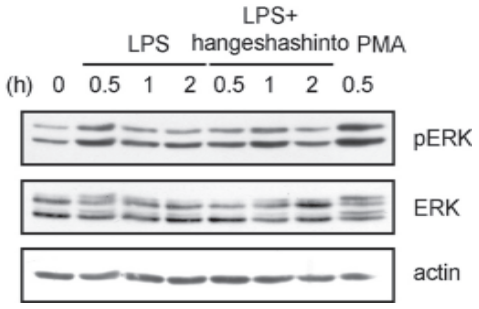

Figure 4. Effect of hangeshashinto on LPS-induced ERK phosphorylation. HGFs were untreated (0 h) or treated with the combinations with LPS (10 ng/ml) and hangeshashinto (0 or 1 mg/ml) for 0.5, 1 and 2 h. For positive control, HGFs were treated with 1 mM of PMA for 0.5 h. Western blotting was performed using anti-phosphorylated ERK or anti-ERK antibodies. pERK: phosphorylated ERK. Upper band indicates ERK1 (p44 MAPK) and lower band ERK2 (p42 MAPK).

ERK [Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody, 1:1,000 dilution] were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Statistical analysis

Differences between control group and experimental groups were evaluated by Dunnett method (Table 2). Differences between groups were evaluated by the pairwise comparison test corrected with Holm method (4 null hypotheses, without LPS vs. with LPS; 3+3 null hypotheses without hangeshashinto vs. with 0.01, 0.1 and 1 mg/ml of hangeshashinto in the absence or presence of LPS; total 10 null hypotheses; Figs. 1B, 1C and 2). All computations were performed with the statistical program R (http://www.r-project.org/). Dunnett method was performed using 'glht' function in 'multcomp' package. 50% inhibitory concentration (IC_{50}) and its 95% confidential interval were calculated using 'ED' function in 'drc' package. Values with $P < 0.05$ were considered as significantly different.

Results

The effect of hangeshashinto on HGFs proliferation

First, we examined the effect of hangeshashinto on HGFs proliferation. In the absence of presence of LPS, hangeshashinto significantly suppressed HGFs proliferation in the dose-dependent manner (Fig. 1A and 1B). In particular, HGFs treated with 1 mg/ml of hangeshashinto were almost dead at day 7. Moreover, HGFs proliferation was slightly but significantly suppressed by LPS treatment (Fig. 1B).

The effect of hangeshashinto on PGE_2, IL-6 and IL-8 production

We examined whether hangeshashinto affects the productions of PGE_2 and inflammatory cytokines (IL-6 and IL-8) by HGFs. Because hangeshashinto affects cell viability, the concentrations of PGE_2, IL-6 and IL-8 were needed for adjustment by the viable cell numbers. When HGFs were treated with LPS and hangeshashinto for 24 h, the viability of HGFs were hardly affected (Fig. 1C).

HGFs without any treatment produced low level of PGE_2. Hangeshashinto significantly suppressed LPS-induced PGE_2 production in a dose-dependent manner (Fig. 2A). IC_{50} of hangeshashinto for LPS-induced PGE_2 production was 0.019 mg/ml and its 95% confidential interval was 0.010-0.028 mg/ml. However, hangeshashinto had little effect on PGE_2 production in the absence of LPS.

In the absence of LPS, IL-6 and IL-8 productions were not altered at 0.01 and 0.1 mg/ml of hangeshashinto but increased at 1 mg/ml of hangeshashinto (Fig. 2B and C). In contrast, hangeshashinto weakly decreased LPS-induced IL-6 and IL-8 productions (Fig. 2B and C).

The effect of hangeshashinto on COX activities

Because PGE_2 production was regulated by COXs and suppressed by acid NSAIDs such as aspirin and diclofenac sodium, which inhibit COXs activities, we examined whether hangeshashinto inhibits COX-1 and COX-2 activities. Hangeshashinto decreased both COX-1 and COX-2 activities to approximately 60% (Table 2).

The effect of hangeshashinto on molecular expressions in arachidonic acid cascade

We examined whether hangeshashinto affects the molecular expression in arachidonic acid cascade. $cPLA_2$ is the most upstream enzyme in arachidonic acid cascade and releases arachidonic acid from plasma membrane. Hangeshashinto decreased $cPLA_2$ expression in the absence or presence of LPS (Fig. 3). COX-2 was not detected in the absence of LPS, and induced by LPS treatment. LPS-induced COX-2 expression was increased with up to 0.1 mg/ml of hangeshashinto and slightly decreased with 1 mg/ml of hangeshashinto (Fig. 3). Annexin1, also named as lipocortin1, is the anti-inflammatory mediator produced by glucocorticoids and inhibits the $cPLA_2$ activity[19,20]. However, both LPS and hangeshashinto showed no effect on annexin1 expression (Fig. 3).

The effects of hangeshashinto 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 hangeshashinto suppresses LPS-induced ERK phophorylation. ERK phosphorylation was enhanced 0.5 h after LPS treatment and thereafter was attenuated, and 1 mg/ml of hangeshashinto weakly suppressed LPS-induced ERK phosphorylation at 0.5 h to 2 h (Fig. 4).

Discussion

In the present study, we examined the effect of hangeshashinto in LPS-treated HGFs. Hangeshashinto suppressed LPS-induced PGE_2 production, but to a lesser extent IL-6 and IL-8, by HGFs as well as shosaikoto and orento[3,4]. It is widely known that PGE_2 leads to inflammatory responses such as vasodilation, enhanced vascular permeability and pain generation. Acid NSAIDs shows anti-inflammatory effect by suppression of PGE production even though they did not affect IL-6 and IL-8 productions. The findings that hangeshashinto suppresses LPS-induced PGE2 production to a basal level suggest that hangeshashinto also has anti-inflammatory effects in periodontal disease and that its effects are mainly mediated by suppression of PGE_2 production.

In the present study, the findings that hangeshashinto suppressed $cPLA_2$ expression, LPS-induced COX-2 expression and COX-2 activity were obtained. However, hangeshashinto suppressed COX-2 activity to approximately 60% at 1 mg/ml. Moreover, hangeshashinto weakly suppressed LPS-induced ERK phosphorylation. Because phosphorylated ERK activates $cPLA_2$ through its phosphorylation[21,22], it is suggested that hangeshashinto suppresses LPS-induced $cPLA_2$ activation. However, the extent may be low. Therefore, the anti-inflammatory effect of hangeshashinto is assumed to be mainly the suppression of $cPLA_2$ expression and COX-2 expression, and to a lesser extent, the suppression of COX-2 activity and $cPLA_2$ activation.

In general, steroidal anti-inflammatory drugs (SAIDs) suppress $cPLA_2$, COX-2 and inflammatory cytokines (such as IL-6 and IL-8) and induce annexin1. In the present study, indeed, hangeshashinto suppresses the expression of $cPLA_2$ and LPS-induced COX-2 expression. However, hangeshashinto shows no or little effect on IL-6 and IL-8 productions and annexin1 expression, suggesting that the mechanism of hangeshashinto for the decrease of PGE_2 production is different from those of SAIDs.

Next, we discuss the difference of the ingredients and the effects between hangeshashinto and orento. The ingredients of hangeshashinto and orento are very similar but the two components are distinct: (1) Hangeshashinto contains ogon instead of keihi,

and (2) the oren content in hangeshashinto (5.4%) is less than one-half of that in orento (12.5%). Hangeshashinto suppressed COX-2 expression, COX-2 activity and $cPLA_2$ expression, while orento increased COX-2 and $cPLA_2$ expressions and did not affect COX-2 activity[4]. These results suggest that ogon suppresses COX-2 expression and activity.

Indeed, ogon contains several flavonoids such as baicalin, baicalein and wogonin[23], and these flavonoids suppress COX-2 expression in several cells[23-25]. Therefore, hangeshashinto suppresses COX-2 expression as well as shosaikoto[3].

Previously, hangeshashinto is reported to inhibit COX-2 activity although which the herbs in hangeshashinto inhibit COX-2 activity remain unclear[11]. The comparison of these two kampo

medicines suggests that ogon inhibits COX-2 activity. However, the components remain to be elucidated. Moreover, ogon may suppresses $cPLA_2$ expression. However, its possibility is low because shosaikoto which also contains ogon did not affect $cPLA_2$ expression[3].

We demonstrated that orento suppresses LPS-induced ERK phosphorylation and further $cPLA_2$ phosphorylation, and suggested that oren, kankyo and/or keihi may have its action[4]. Hangeshashinto also suppressed LPS-induced ERK phosphorylation, but its extent was weak. Considering the difference of oren content in hangeshashinto and orento, it is suggested that oren suppresses LPS-induced ERK phosphorylation. In fact, berberine, which is the main component of oren, is reported to suppress ERK phosphorylation in vascular smooth muscle cell[26] and mouse macrophages[27]. Because hangeshashinto suppressed LPS-induced ERK phosphorylation weakly, the inhibitory action of hangeshashinto on $cPLA_2$ activation is assumed to be weak compared to orento.

Many studies demonstrated that NSAIDs administration prevents gingival inflammation (reviewed in ref. 2). And several clinical studies indicated that the concentration of PGE_2 in gingival crevicular fluid (GCF) is increased in periodontal disease[28] and is decreased by oral administration or mouse wash of NSAIDs[29,30]. Considering the facts that both NSAIDs and hangeshashinto suppress PGE_2 production, it is possible that administration of hangeshashinto also decreases PGE_2 concentration in GCF and results in the improvement of gingival inflammation. Therefore, hangeshashinto may be useful for the improvement of gingival inflammation in periodontal disease. Importantly, hangeshashinto did not affect basal level of PGE_2. Because PGE_2 produced by COX-1 protects gastric mucosa, these results suggest that hangeshashinto may have minimal gastrointestinal dysfunction.

In summary, we demonstrated that hangeshashinto suppresses $cPLA_2$ and COX-2 expressions and to a lesser extent COX-2 activity, and results in PGE_2 production by HGFs. Hangeshashinto may be useful for the improvement of inflammation in periodontal disease.

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