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

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Received August 28, 2009; accepted December 17, 2009; published online January 8, 2010

In the present study, we investigated the effects of a Kampo medicine Orento (TJ-120) on the production of prostaglandin E_2 (PGE_2), interleukin (IL)-6 and IL-8 by human gingival fibroblasts (HGFs) treated with lipopolysaccharide from *Porphyromonas gingivalis* (PgLPS). HGFs proliferation was dose-dependently decreased with Orento at days 3 and 7. However, treatment with PgLPS (10 ng/ml), Orento (up to 1 mg/ml) and their combinations for 24 h did not affect the viability of HGFs. Orento suppressed PgLPS-induced PGE_2 production in a dose-dependent manner but did not alter basal PGE_2 level. In contrast, Orento did not alter PgLPS-induced IL-6 and IL-8 productions. These alterations by Orento were similar to those by a mitogen-activated protein kinase kinase (MAPKK/MEK) inhibitor, PD98059. A Orento showed no effect on cyclooxygenase (COX)-1 and COX-2 activities, and increased cytoplasmic phospholipase A_2 ($cPLA_2$) expression and increased PgLPS-induced COX-2 expression. Orento suppressed PgLPS-induced mobility retardation of $cPLA_2$ band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, that is $cPLA_2$ phosphorylation and its activation, while Orento alone did not alter $cPLA_2$ phosphorylation. Orento suppressed PgLPS-induced extracellular signal-regulated kinase (ERK) phosphorylation, which is known to lead to ERK activation and $cPLA_2$ phosphorylation. These results suggest that Orento decreased PGE_2 production by inhibition of $cPLA_2$ phosphorylation and its activation *via* inhibition of ERK phosphorylation, and also that Orento may be useful to improve gingival inflammation in periodontal disease.

Key words Orento; prostaglandin E_2; anti-inflammatory effect; human gingival fibroblast

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) suppressed lipopolysaccharide (LPS)-induced prostaglandin E_2 (PGE_2) production by human gingival fibroblasts (HGFs) through the suppression of both cyclooxygenase-2 (COX-2) activity and expression, and suggested that Shosaikoto has an anti-inflammatory effect in periodontal disease and the possibility of less gastrointestinal dysfunction.[3)] Other kampo medicine Orento (TJ-120) has been used for inflammatory diseases such as acute gastritis and stomatitis.[4)] From these indications, we considered that Orento could be useful for periodontal disease. Although Orento has been shown to inhibit the formation of calcium phosphate precipitates,[5)] there is few studies about anti-inflammatory effect. Therefore, it is of interest to examine the precise anti-inflammatory mechanism of Orento.

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.[6—9)] 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[10)] and IL-6 and IL-8[11)] in the presence of LPS, these mediators and cytokines in periodontal tissues are thought to be 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 Orento on LPS-induced PGE_2, IL-6 and IL-8 productions using this *in vitro* model.

MATERIALS AND METHODS

Reagents Powder of Orento was obtained from Tsumura & Co. (Tokyo, Japan), and its components were indicated in Table 1. Orento was suspended in Dulbecco's modified Eagle's medium (D-MEM, Sigma, St. Louis, MO, U.S.A.) 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 0.45 μm-pore membrane. LPS from *Porphyromonas gingivalis* 381 (PgLPS) was provided by Drs. Tatsuji Nishihara and Nobuhiro Hanada (National Institutes of Public Health, Wako, Japan). Phorbol 12-myristate 13-acetate (PMA) and PD98059 [mitogen-activated protein kinase kinase (MAPKK/MEK) inhibitor] were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). Arachidonic acid solution (from porcine liver) was purchased from Sigma. The anti-

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Table 1. The Ingredient of Orento Formula

Japanese name	Latin name	Amount [g (percent)]
Hange	*Pinelliae tuber*	6.0 (25.0)
Ouren	*Coptidis rhizoma*	3.0 (12.5)
Kankyo	*Zingiberis siccatum*	3.0 (12.5)
Kanzo	*Glycyrrhizae radix*	3.0 (12.5)
Keihi	*Cinnamomi cortex*	3.0 (12.5)
Taiso	*Zizyphi fructus*	3.0 (12.5)
Ninjin	*Ginseng radix*	3.0 (12.5)
	Total	24.0 (100)

bodies against cyclooxygenase-2 (COX-2, sc-1745), cytoplasmic phospholipase A_2 ($cPLA_2$, sc-438), annexin I (sc-11387) and actin (sc-1616), which detects a broad range of actin isoforms, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and the antibodies against extracellular signal-regulated kinase (ERK; p44/42 MAP kinase antibody) and phosphorylated ERK [Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody] were from Cell Signaling Technology (Danvers, MA, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

Cells HGFs were prepared as described previously.[12] HGFs were maintained in Dulbecco's modified Eagle's medium (D-MEM; Sigma, St. Louis, MO, U.S.A.) containing 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C 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 our institution. Informed consent was obtained from each subject for the collection of HGFs.

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, 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_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, U.S.A.), and background value was subtracted from each value.

Cytokine Measurement by Enzyme-Linked Immunosorbent Assay (ELISA) HGFs (10000 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 Orento (0, 0.01, 0.1, 1 mg/ml) in the absence or presence of PgLPS (10 ng/ml) for 24 h (200 μl per each well). In some experiments, 10 μM of arachidonic acid was added simultaneously. The numbers of cells were measured using WST-8. The concentrations of IL-6, IL-8 and PGE_2 in the culture supernatants were measured by ELISA according to the manufacturer's instructions (IL-6 and IL-8, Biosource International Inc., Camarillo, CA, U.S.A.; PGE_2, Cayman Chemical, Ann Anbor, MI, U.S.A.), and were adjusted by the number of remaining cells.

Measurement of Cyclooxygenase Activity The effects of Orento on the activities of COX-1 and COX-2 were analyzed using COX inhibitor screening assay (Cayman Chemical, Ann Anbor, MI, U.S.A.) according to the manufacturer's instructions. COXs activities were evaluated by the measurement of prostaglandin produced from arachidonic acid by COX-1 or COX-2 in duplicate. These values were normalized to a relative value of 100% for the cells without both PgLPS and Orento treatment.

Table 2. Effect of Orento on COX Activities

Concentration (mg/ml)	COX activity (%)	
	COX-1	COX-2
0	100.0±6.1	100.0±3.8
0.1	105.2±3.2	108.5±6.0
1	116.0±4.3	107.4±4.8

Data represent the mean±S.D. (n=3). Not significant by Dunnett's method.

Western Blotting HGFs were cultured in 60 mm dish and treated with the combinations of PgLPS and Orento. After 24 h, cells were washed twice with phosphate buffered saline, transferred into microcentrifuge tubes, and centrifuged at 6000×***g*** for 5 min at 4 °C. 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 ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-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 12000×***g*** for 15 min at 4 °C and supernatants were collected. The protein concentration was measured using BCA Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL, U.S.A.).

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).

Statistical Analysis Data are presented as means±standard deviation (S.D.). 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 PgLPS *vs.* with PgLPS; 3+3 null hypotheses without Orento *vs.* with 0.01, 0.1 and 1 mg/ml of Orento in the absence or presence of PgLPS; total 10 null hypotheses; Figs. 1B, C and 2). All computations were performed with the statistical program R[13] and Dunnett method was performed using the package 'multcomp'.[14] Values with $p<0.05$ were considered as significantly different.

RESULTS

The Effect of Orento on HGFs Proliferation We ex-

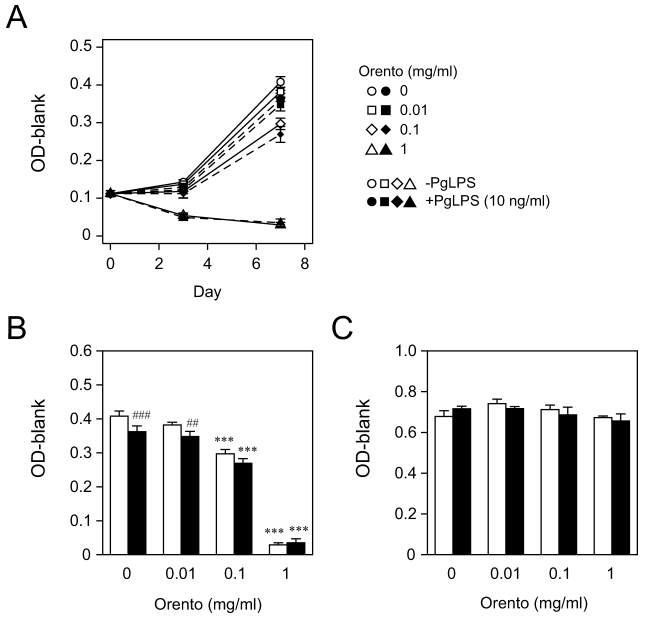

Fig. 1. Effect of Orento on HGFs Proliferation

(A, B) HGFs were plated in 96-well microplate at 2000 cells/ml and were added the media containing PgLPS (0, 10 ng/ml) and Orento (TJ-120; 0, 0.01, 0.1 and 1 mg/ml). Cell numbers were evaluated by WST-8 at day 0, 3 and 7 (A) and at day 7 (B). (C) The effect of Orento on viability of HGFs at 24 h. HGFs were plated in 96-well microplate at 10000 cells/ml and were added the media containing PgLPS and Orento. Cell numbers were evaluated by WST-8 at 24 h. Open bars, treatment without PgLPS; closed bars, treatment with 10 ng/ml of PgLPS. $^{***}p<0.001$ (without Orento *vs.* with Orento). $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ (without PgLPS *vs.* with PgLPS). p values were calculated by pairwise comparison and corrected with Holm method (10 null hypotheses).

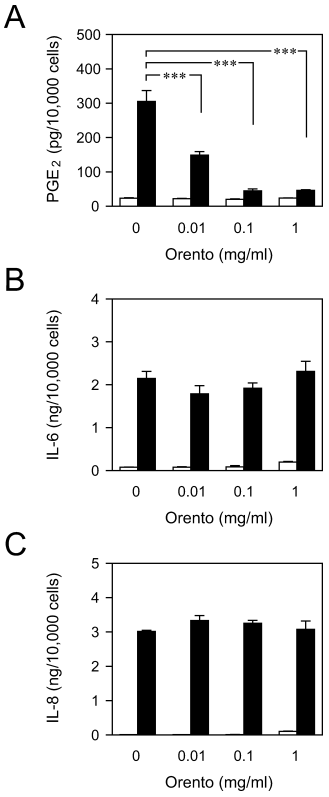

Fig. 2. Effects of Orento on the Production of IL-6, IL-8 and PGE_2

HGFs were treated with the combinations with PgLPS (0, 10 ng/ml) and Orento (TJ-120; 0, 0.01, 0.1 and 1 mg/ml) for 24 h, and the concentrations of IL-6 (A), IL-8 (B) and PGE_2 (C) were measured by ELISA. The concentrations were adjusted by the cell numbers and expressed as per 10000 cells. Open bars, treatment without PgLPS; closed bars, treatment with 10 ng/ml of PgLPS. $^{***}p<0.001$. p values were calculated by pairwise comparison and corrected with Holm method (10 null hypotheses).

amined the effect of Orento on HGFs proliferation. In the absence of presence of PgLPS, Orento suppressed HGFs proliferation in the dose-dependent manner (Figs. 1A, B). In particular, HGFs treated with 1 mg/ml of Orento were almost dead at day 7. Moreover, HGFs proliferation was slightly suppressed by PgLPS treatment (Fig. 1B). These results indicate that 1 mg/ml of Orento shows a cytotoxicity.

The Effect of Orento on PGE_2, IL-6 and IL-8 Production We examined whether Orento affects the productions of PGE_2 and inflammatory cytokines (IL-6 and IL-8) by HGFs. Because Orento affects cell viability, the concentrations of PGE_2, IL-6 and IL-8 were needed for adjustment by the results of WST-8. When HGFs were treated with PgLPS and Orento for 24 h, the viability of HGFs were hardly affected (Fig. 1C).

HGFs without any treatment produced low level of PGE_2. When HGFs were treated with PgLPS, HGFs produced significant level of PGE_2. Orento suppressed PgLPS-induced PGE_2 production in a dose-dependent manner (Fig. 2A). However, Orento had little effect on PGE_2 production in the absence of PgLPS.

In the absence of PgLPS, Orento did not affect IL-6 and IL-8 production (Figs. 2B, C). When HGFs were treated with 10 ng/ml of PgLPS, HGFs produced large amount of IL-6 and IL-8. Orento did not affected PgLPS-induced IL-6 and IL-8 productions (Figs. 2B, C). These results indicate that Orento suppressed PgLPS-induced production PGE_2 by HGFs but shows little effect on IL-6 and IL-8 productions.

The Effects of Orento 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 Orento inhibits COX-1 and COX-2 activities. However, up to 1 mg/ml of Orento did not affect both COX-1 and COX-2 activities (Table 2).

The Effects of Orento on Molecular Expressions in Arachidonic Acid Cascade We examined whether Orento affects the molecular expression in arachidonic acid cascade. COX-2 was not detected in the absence of PgLPS, and induced by PgLPS treatment. PgLPS-induced COX-2 expression was increased with up to 0.1 mg/ml of Orento and slightly decreased with 1 mg/ml of Orento (Fig. 3).

$cPLA_2$ is the most upstream enzyme in arachidonic acid cascade and releases arachidonic acid from plasma membrane. Orento increased $cPLA_2$ expression in a dose-dependent manner in the absence or presence of PgLPS (Fig. 3). In contrast, other PLA_2 isoforms, calcium-independent PLA_2 ($iPLA_2$) and secretory PLA_2 ($sPLA_2$), were not detected (data not shown), indicating that $cPLA_2$ is a major isoform which release arachidonic acid in HGFs.

Annexin 1, also named as lipocortin 1, is the anti-inflammatory mediator produced by glucocorticoids and inhibits the $cPLA_2$ activity.[15,16] However, both PgLPS and Orento showed no effect on annexin 1 expression (Fig. 3).

The Effects of Orento on $cPLA_2$ Phosphorylation and ERK Phosphorylation $cPLA_2$ activity were regulated by its phosphorylation at Ser505[17,18] and its phosphorylation causes mobility retardation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.[19] Then,

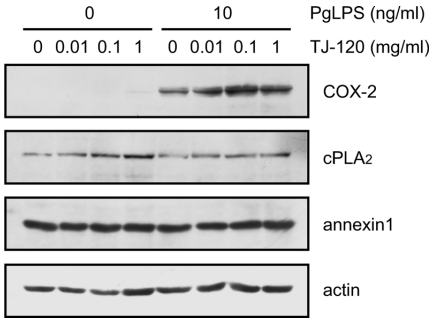

Fig. 3. Effects of Orento on COX-2, $cPLA_2$ and Annexin 1 Expressions

HGFs were treated with the combination of PgLPS and Orento for 8h, and protein levels were examined by Western blotting.

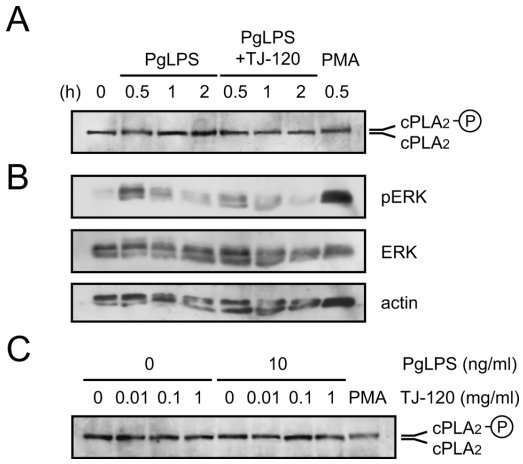

Fig. 4. Effect of Orento on PgLPS-Induced $cPLA_2$ Phosphorylation and ERK Phosphorylation

(A, B) HGFs were untreated (0 h) or treated with the combinations with PgLPS (10 ng/ml) and Orento (0 or 1 mg/ml) for 0.5, 1 and 2 h. For positive control, HGFs were treated with 1 μM of PMA for 0.5 h. Western blotting was performed using anti-$cPLA_2$ (A), anti-phosphorylated ERK or anti-ERK antibodies (B). $cPLA_2$: non-phosphorylated $cPLA_2$, $cPLA_2$-P: phosphorylated $cPLA_2$, pERK: phosphorylated ERK. Upper band indicates ERK1 (p44 MAPK) and lower band ERK2 (p42 MAPK). (C) HGFs were treated with the combinations with PgLPS (0, 10 ng/ml) and Orento (0, 0.01, 0.1, 1 mg/ml) for 2 h. Western blotting was performed using anti-$cPLA_2$ antibody.

we examined whether Orento affects $cPLA_2$ phosphorylation. First, we examined PgLPS-induced $cPLA_2$ phosphorylation in the absence or presence of Orento (1 mg/ml). $cPLA_2$ was phosphorylated at 0.5 and 1 h after PgLPS treatment with or without of Orento. At 2 h after treatment, $cPLA_2$ was phosphorylated with PgLPS alone but was not phosphorylated with the combination of PgLPS and Orento (Fig. 4A). These results indicated that Orento suppressed PgLPS-induced $cPLA_2$ phosphorylation at 2 h after treatment (Fig. 4A).

$cPLA_2$ is reported to be phosphorylation and activated by extracellular signal-regulated kinase (ERK) directly.[17] Previously, we examined the effect of an ERK inhibitor, PD98059, on PgLPS-induced IL-8 production by HGFs and obtained the results similar to Orento.[20] Then, we again examined the effect of PD98059 on PGE_2, IL-6 and IL-8 productions (Table 3). PD98059 did not affected a basal level of PGE_2 but decreased PgLPS-induced PGE_2 production. Basal levels of IL-6 and IL-8 productions were increased by PD98059. PD98059 slightly increased PgLPS-induced IL-6 production but not IL-8. These results indicate that the effects of Orento on PGE_2, IL-6 and IL-8 productions are overall similar to those of PD98059, and prompted us to examine whether

Table 3. Effect of ERK Inhibitor on PGE_2, IL-6 and IL-8 Productions

		DMSO	PD98059
PGE_2 (pg/10000 cells)	−PgLPS	42.0±8.6	23.4±1.5
	+PgLPS	226.8±29.9	27.7±2.3
IL-6 (ng/10000 cells)	−PgLPS	0.02±0.00	0.21±0.02
	+PgLPS	1.89±0.38	2.65±0.39
IL-8 (ng/10000 cells)	−PgLPS	n.d.	0.13±0.02
	+PgLPS	2.48±0.28	2.26±0.39

HGFs were pretreated with DMSO or 20 μM PD98059 for 60 min, and then treated with the combination of 10 ng/ml of PgLPS and DMSO or 20 μM PD98059 for 24 h. PGE_2, IL-6 and IL-8 levels in supernatants were measured by ELISA and were adjusted by the cell numbers and expressed as per 10000 cells. n.d.: not detected.

Table 4. Effect of Exogenous Arachidonic Acid on PGE_2 Production

	PGE_2 (pg/10000 cells)	
	(−)	AA (10 μM)
(−)	24.2±2.7	229.4±18.4
Orento (1 mg/ml)	25.6±2.3	331.8±41.0
PgLPS (10 ng/ml)	356.6±40.4	945.5±103.0
PgLPS+Orento	41.0±5.2	1002.4±49.1

HGFs were treated with the combination of 10 ng/ml of PgLPS, Orento (1 mg/ml) and arachidonic acid (AA; 10 μM) for 24 h. PGE_2 level in supernatants were measured by ELISA and were adjusted by the cell numbers and expressed as per 10000 cells.

Orento suppresses PgLPS-induced ERK phosphorylation. ERK phosphorylation was enhanced 0.5 h after PgLPS treatment and thereafter was attenuated, and 1 mg/ml of Orento suppressed PgLPS-induced ERK phosphorylation at 0.5 to 2 h (Fig. 4B).

Next, we examined $cPLA_2$ phosphorylation at 2 h after PgLPS and Orento treatment. Without PgLPS treatment, $cPLA_2$ was not phosphorylated (Fig. 4C). When HGFs were treated with only PgLPS or both PgLPS and 0.01 mg/ml of Orento, $cPLA_2$ was phosphorylated. However, when HGFs were treated with both PgLPS and 0.1 or 1 mg/ml of Orento, $cPLA_2$ was not phosphorylated (Fig. 4C). These results indicate that Orento dose-dependently suppresses PgLPS-induced $cPLA_2$ phosphorylation in HGFs.

The Effects of Orento on the Downstream Molecules of Arachidonic Acid Cascade Finally, we confirmed whether the suppression of PGE_2 production by Orento is mediated on the downstream molecules of arachidonic acid cascade. For this purpose, exogenous arachidonic acid was added. Exogenous arachidonic acid increased PgLPS-induced PGE_2 level. Moreover, when HGFs were treated with both PgLPS and Orento, arachidonic acid increased PGE_2 level comparable to that treated with both PgLPS and arachidonic acid (Table 4). These results indicate that Orento did not affect the downstream molecules of arachidonic acid cascade.

DISCUSSION

In the present study, we examined the effect of Orento in PgLPS-treated HGFs. We showed that Orento suppressed PgLPS-induced PGE_2 production, but not IL-6 and IL-8, by HGFs as well as Shosaikoto.[3] It is widely known that PGE_2 leads to inflammatory responses such as vasodilation, enhanced vascular permeability and pain generation. Acid NSAIDs show anti-inflammatory effect by suppression of

PGE_2 production even though they did not affect IL-6 and IL-8 productions. The findings that Orento suppresses PgLPS-induced PGE_2 production to a basal level suggest that Orento also has anti-inflammatory effects in periodontal disease and that its effects are mainly mediated by suppression of PGE_2, but not IL-6 and IL-8, production.

In the present study, we focused on the effect of Orento on $cPLA_2$ function and demonstrated that Orento suppresses PgLPS-induced $cPLA_2$ phosphorylation (namely, activation). Considering that Orento did not affect COXs activities, that Orento did not suppress COX-2 and $cPLA_2$ expression, did not increase annexin 1 expression, and that the suppression of PGE_2 production by Orento was canceled by the addition of exogenous arachidonic acid, it is suggested that the action mechanism of Orento is suppression of arachidonic acid release through the suppression of PgLPS-induced $cPLA_2$ activation but not downstream of arachidonic acid cascade, which includes microsomal prostaglandin E synthase-1 (mPGES-1) converting PGH_2 to PGE_2.

The mechanism of Orento is thought to be different from those of generally used anti-inflammatory drugs, acid NSAIDs and steroidal anti-inflammatory drugs (SAIDs). Acid NSAIDs inhibit COXs activities, while Orento showed no inhibitory effect on both COX-1 and COX-2 activities (Table 2). SAIDs suppress $cPLA_2$, COX-2 and inflammatory cytokines (such as IL-6 and IL-8) and induce annexin 1, which inhibits $cPLA_2$ activity. In fact, dexamethasone suppressed PgLPS-induced IL-6 and IL-8 productions as well as PGE_2 production (data not shown). However, Orento showed little or no effect on the expression of these molecules (Fig. 3). These results suggest that Orento has a novel anti-inflammatory mechanism.

The arachidonic acid release by $cPLA_2$ is regulated by two mechanisms. The first is Ser505 phosphorylation[19] and the second is $cPLA_2$ localization to membrane or phospholipid vesicles elicited by increased intracellular Ca^{2+} concentration.[21—23] Because Orento showed little effect on a basal level of PGE_2, the mechanism of Orento is suggested to suppress $cPLA_2$ phosphorylation (Fig. 4C) but not to inhibit $cPLA_2$ activity itself. Next, we discuss whether the suppression of $cPLA_2$ phosphorylation can explain for the alteration of PGE_2 production by Orento. (i) When HGFs were not treated or treated with only Orento, because $cPLA_2$ was not phosphorylated ($cPLA_2$ activity is basal level) and COX-2 was not expressed (Fig. 3), HGFs slightly release arachidonic acid and produce a small mount of PGE_2 mediated by COX-1. (ii) When HGFs were treated with only PgLPS or both PgLPS and low dose of Orento, both $cPLA_2$ phosphorylation ($cPLA_2$ activation) and COX-2 expression were observed (Figs. 3, 4C), resulting in production of large amount of PGE_2. (iii) When HGFs were treated with both PgLPS and high dose of Orento, COX-2 expression, but not $cPLA_2$ phosphorylation, was observed (Figs. 3, 4C). PgLPS-induced PGE_2 productions with 0.1 or 1 mg/ml of Orento were similar, but both were higher than those with only Orento (Fig. 2A). These results suggest that COX-2 produce PGE_2 from arachidonic acid released by $cPLA_2$ with basal level of activity, and therefore may explain the finding that Orento failed to suppress PGE_2 production to the level without PgLPS. Moreover, It is reported that phosphorylation of $cPLA_2$ increases its activity by 2- to 3-fold.[19] This increased activity ratio is similar to the ratio of PGE_2 level with PgLPS to that without PgLPS (Fig. 2A). From these findings, we consider that the suppression of $cPLA_2$ phosphorylation by Orento explains for the alteration of PGE_2 production.

We also demonstrated that the alteration of PGE_2, IL-6 and IL-8 levels by Orento are quite similar to those by PD98059 (Fig. 2, Table 3). Moreover, ERK phosphorylates and activates $cPLA_2$.[17,24] Indeed, Orento suppressed PgLPS-induced ERK phosphorylation (Fig. 4B). These results suggest that the effect of Orento on $cPLA_2$ phosphorylation is mediated by the modulation of ERK activation. Which components in Orento phosphorylate ERK and activate $cPLA_2$. In our previous report, Shosaikoto did not affect basal levels of IL-6 and IL-8,[3] suggesting that Shosaikoto contains no components which inhibit ERK phosphorylation. Shosaikoto are composed of Saiko, Hange, Ogon, Taiso, Ninjin, Kanzo and Shokyo. Therefore, the components that are contained in only Orento (Oren, Kankyo and/or Keihi) may suppress $cPLA_2$ phosphorylation and PGE_2 production.

In contrast, it is reported that LPS failed to increase intracellular Ca^{2+} concentration in rat liver macrophage for at least 24 h.[25] These results suggest that LPS does not translocate $cPLA_2$ to membrane or phospholipid vesicles and, therefore, that Orento may not be involved in translocation of $cPLA_2$.

To date, there is no drug that suppresses $cPLA_2$ activity among various anti-inflammatory drugs used generally. Therefore, Orento is an anti-inflammatory drug possessing a mechanism different from conventional drugs. Recently, the concentration of leptin, which is mainly synthesized in adipocytes and regulates weight control,[26] in gingival crevicular fluid is negatively correlated with periodontal disease progression.[27—29] Moreover, leptin is reported to suppress PgLPS-induced $cPLA_2$ activation in salivary gland acinar cells[30] and *Helicobacter pylori* LPS-induced $cPLA_2$ activation in gastric mucosal cells.[31] However, leptin is also reported to increase COX-2 expression and PGE_2 production in human endometrial cancer cells.[32] Therefore, leptin may be difficult to use an anti-inflammatory drugs.

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[33] and is decreased by oral administration or mouse wash of NSAIDs.[34,35] Considering the facts that both NSAIDs and Orento suppress PGE_2 production, it is possible that administration of Orento also decreases PGE_2 concentration in GCF and results in the improvement of gingival inflammation. Therefore, Orento may be useful for the improvement of gingival inflammation in periodontal disease. Importantly, Orento did not alter COX-1 activity and basal level of PGE_2. Because PGE_2 produced by COX-1 protects gastric mucosa, these results suggest that Orento may have minimal gastrointestinal dysfunction.

In summary, we demonstrated that Orento suppresses PgLPS-induced ERK phosphorylation and following $cPLA_2$ phosphorylation and results in PGE_2 production by HGFs. Orento may be useful for the improvement of inflammation in periodontal disease.

Acknowledgments We thank Drs. Tatsuji Nishihara and

Nobuhiro Hanada for providing *Porphyromonas gingivalis* LPS. We also thank Associate Prof. Takashi Uematsu (Department of Oral and Maxillofacial Surgery) for HGFs preparation, Mr. Takashi Ogasahara (Tsumura & Co.) for the information of Orento and Keiko Fujii for technical assistance. The study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (Code No. 18791390) of Japan and a Scientific Research Special Grant from Matsumoto Dental University.

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