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

Toshiaki Ara,[a,b] Yoshihiro Maeda,[b] Yoshiaki Fujinami,[a] Yasuhiro Imamura,[a,b] Toshimi Hattori,[a,b] and Pao-Li Wang*,[a,b]

[a] *Department of Pharmacology, Matsumoto Dental University; and* [b] *Department of Hard Tissue Research, Graduate School of Oral Medicine, Matsumoto Dental University; 1780 Gobara, Hirooka, Shiojiri, Nagano 399–0781, Japan.*

In the present study, we investigated the anti-inflammatory effects of a Kampo medicine Shosaikoto (TJ-9) using *in vitro* periodontal disease model, in which human gingival fibroblasts (HGFs) treated with lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (PgLPS) produce IL-6, IL-8 and prostaglandin E_2 (PGE_2). Treatment with PgLPS (10 ng/ml), TJ-9 (up to 1 mg/ml) and their combinations for 24 h did not affect the viability of HGFs. Moreover, TJ-9 did not alter LPS-induced IL-6 and IL-8 productions. However, TJ-9 significantly suppressed LPS-induced PGE_2 production in a dose-dependent manner but TJ-9 alone did not affect basal PGE_2 level. Western blotting demonstrated that TJ-9 decreased cyclooxygenase-2 (COX-2) expression in a dose-dependent manner but not phospholipase A_2. Moreover, TJ-9 selectively and dose-dependently inhibited COX-2 activity. These results suggest that TJ-9 decreased PGE_2 production by inhibition of both COX-2 expression and activity and that TJ-9 may be useful to improve gingival inflammation in periodontal disease.

Key words Shosaikoto; periodontal disease; gingival fibroblast; lipopolysaccharide

Periodontal disease is one of major oral diseases and is thought to be biofilm infectious disease.[1] Periodontal disease comprises a group of infections that leads to inflammation of the gingiva and destruction of periodontal tissues, and is accompanied by alveolar bone loss in severe clinical cases. The tissue destruction is the result of activation of the host's immuno-inflammatory response to virulence factors[2] such as lipopolysaccharide (LPS) and peptidoglycan. The elimination of these virulence factors by initial preparation is very important for the treatment of periodontal disease. In some case, 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 (summarized in ref. 2). However, acid NSAIDs are reported to have side effects such as gastrointestinal dysfunction.

Shosaikoto (TJ-9) is one of the Kampo medicines and has been used clinically to treat various inflammatory diseases including chronic hepatitis. In addition, TJ-9 protects the liver plasma membrane in endotoxin-treated mice.[3] TJ-9 inhibits LPS-induced tumor necrosis factor (TNF)-α production by macrophages[4] and pretreatment with TJ-9 prevents septic shock in LPS-injected rats.[5] TJ-9 also inhibits prostaglandin E_2 (PGE_2) production by zymosan-treated monocytes.[6] Moreover, TJ-9 suppresses liver inflammatory responses and fibrosis in dimethylnitrosamine-induced liver injury rats.[7] And, TJ-9 induces apoptosis of a human hepatocellular carcinoma cell line and a cholangiocarcinoma cell line,[8] and prevents the development of hepatocellular carcinoma.[9] Considering these reports, we assumed that TJ-9 has an ability to suppress gingival inflammation in periodontal disease.

Human gingival fibroblasts (HGFs) are the most prominent cells in periodontal tissue. And HGFs produce inflammatory cytokines such as interleukin (IL)-6 and IL-8 and eicosanoids such as PGE_2 when HGFs were treated with LPS.[10—12] 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 IL-6 and IL-8 in the presence of LPS,[13] we consider that the examinations of effect on HGFs, as well as monocytes and macrophages, are important in the study on periodontal disease. In the present study, we examined anti-inflammatory effects of TJ-9 in this model using HGFs.

MATERIALS AND METHODS

Reagents Shosaikoto (TJ-9) was obtained from Tsumura & Co. (Tokyo, Japan). TJ-9 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. *Porphyromonas gingivalis* LPS (PgLPS) was provided by Drs. Tatsuji Nishihara and Nobuhiro Hanada (National Institutes of Public Health, Wako, Japan). The antibodies against cytoplasmic phospholipase A_2 (cPLA$_2$, sc-438), cyclooxygenase-2 (COX-2, sc-1745) and actin (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

Cells HGFs were prepared from explants of human normal gingival tissues as described previously,[14] and were maintained in culture medium in a humidified atmosphere of 5% CO_2 at 37 °C. HGFs were used between 10th to 20th passages in the assays. The present study was approved by the Ethical Committee of our institution. Informed consent was obtained from each subject.

MTT Assay The numbers of cells were measured by MTT assay. In brief, the media were removed by aspiration and the cells were treated with 0.5 mg/ml dimethylthiazol-2-

yl-2,5-diphenyltetrazolium bromide (MTT, Sigma) in culture medium for 4 h at 37 °C. After washed with PBS once, isopropanol/0.04 M HCl was added and OD_{570} were measured and the value of blank is subtracted.

Cytokine Measurement by Enzyme-Linked Immunosorbent Assay (ELISA) HGFs (1×10^4 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 TJ-9 (0 to 1 mg/ml) in the absence or presence of PgLPS (10 ng/ml) for 24 h (200 μl per each well). The numbers of cells were measured using MTT assay. The concentrations of IL-6, IL-8 and prostaglandin E_2 (PGE_2) in the culture supernatants were measured by ELISA according to the manufactures' instructions (IL-6 and IL-8, Biosource International Inc., Camarillo, CA; PGE_2, R&D Systems, Minneapolis, MN, U.S.A.), and were adjusted by the number of remaining cells.

Western Blotting HGFs were cultured in 60 mm dish and treated with the combinations of LPS and TJ-9. After 24 h, cells were washed twice with PBS, transferred into microcentrifuge tubes, and centrifuged at $6000\times\boldsymbol{g}$ 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 EGTA, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 5 μg/ml leupeptin and 1 μg/ml pepstatin) for 30 min at 4 °C. Then, samples were centrifuged at $12000\times\boldsymbol{g}$ 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, Amersham Bioscience, 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 (Amersham Bioscience).

Measurement of Cyclooxygenase Activity The effects of TJ-9 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 manufactures' 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 TJ-9 treatment.

Statistical Analysis Data are presented as means±standard deviation (S.D.). Differences between groups were evaluated using the pairwise comparison test corrected with Holm method (Fig. 2). Differences between control group and test groups were evaluated with Dunnett method (Fig. 3). All computations were performed with the statistical program R (http://www.r-project.org/). Values with $p<0.05$ were considered as significantly different.

RESULTS

The Effects of TJ-9 on IL-6, IL-8 and PGE_2 Productions We examined the effects of TJ-9 in *in vitro* periodontal disease model. TJ-9 was used at 0 to 1 mg/ml because the numbers of HGFs were not decreased at least up to 2 mg/ml after 24 h but decreased at 5 mg/ml (data not shown). First, we examined whether LPS affects viability of HGFs in various concentrations of TJ-9 by MTT assay. No obvious difference was observed in the cell numbers (Fig. 1).

Next, we examined whether TJ-9 affects the productions of inflammatory cytokines (IL-6 and IL-8) and PGE_2 by HGFs. The concentrations of IL-6, IL-8 and PGE_2 were adjusted by the results of MTT assay (Fig. 1). In the absence of PgLPS, TJ-9 did not affect IL-6 and IL-8 production (Figs. 2A, B). When HGFs were treated with 10 ng/ml of PgLPS, HGFs produced large amount of IL-6 and IL-8. TJ-9 did not affected LPS-induced IL-6 and IL-8 productions (Figs. 2A, B).

HGFs without any treatment produced low level of PGE_2. When HGFs were treated with PgLPS, HGFs produced significant level of PGE_2. TJ-9 inhibited LPS-induced PGE_2 production in a dose-dependent manner (Fig. 2C). However, TJ-9 had little effect on PGE_2 production in the absence of PgLPS. The similar results were found for PGE_2 production when HGFs were treated with LPS (data not shown). These results indicated that TJ-9 inhibited LPS-induced production PGE_2 by HGFs but not IL-6 and IL-8.

The Effects of TJ-9 on COX-2 Expression and Activity HGFs were treated with 10 ng/ml of PgLPS for 0 (untreated), 4, 8 and 24 h, and protein levels of $cPLA_2$ and COX-2 were examined by Western blotting. $cPLA_2$ was expressed at 4 and 8 h and COX-2 at 8 h (data not shown). Therefore, we examined the effects of TJ-9 on LPS-induced $cPLA_2$ and COX-2 expressions at 8 h. The levels of $cPLA_2$ were similar regardless of PgLPS and TJ-9. COX-2 was not detected in the absence of PgLPS, and induced by PgLPS treatment. LPS-induced COX-2 expression was decreased with TJ-9 in a dose-dependent manner (Fig. 3A).

We also examined whether TJ-9 inhibits COX-1 and COX-2 activities. Up to 1 mg/ml of TJ-9 did not affected COX-1 activity. TJ-9 did not affected COX-2 activity at 0.1 mg/ml but did at 0.3 and 1 mg/ml (Fig. 3B). These results indicate that TJ-9 selectively inhibited COX-2 activity and that suggested that TJ-9 decreases PGE_2 production by the inhibition
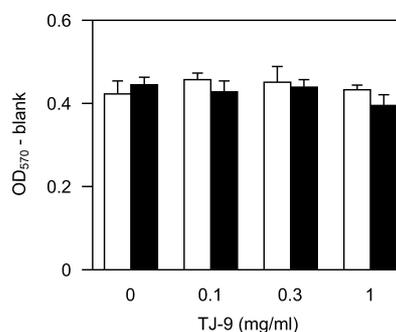

Fig. 1. The Effect of Shosaikoto (TJ-9) on Viability of HGFs

HGFs were treated with the combinations with PgLPS (10 ng/ml) and TJ-9 (0 to 1 mg/ml) for 24 h and were performed to MTT assay. Open bars, treatment without PgLPS; closed bars, treatment with 10 ng/ml of PgLPS.
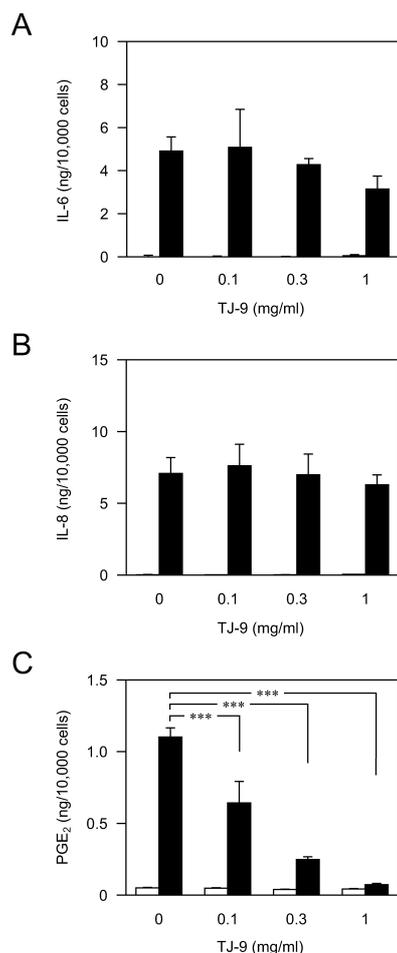
A

B

C

A

PgLPS (ng/ml)   0   10
TJ-9 (mg/ml)   0 0.1 0.3 1   0 0.1 0.3 1

cPLA2

COX-2

actin

B

Fig. 2.   The Effects of Shosaikoto (TJ-9) on the Production of IL-6, IL-8 and PGE$_2$

HGFs were treated with the combinations with PgLPS (10 ng/ml) and TJ-9 (0 to 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. *** $p < 0.001$ *vs.* control corrected by Holm method (7 null hypotheses). Open bars, treatment without PgLPS; closed bars, treatment with 10 ng/ml of PgLPS.

Fig. 3.   (A) The Effects of Shosaikoto (TJ-9) on cPLA$_2$ and COX-2 Expressions

HGFs were treated with the combination of PgLPS and TJ-9 for 8 h, and protein levels were examined by Western blotting.

(B) The Effects of TJ-9 on COXs Activity

* $p < 0.05$ and *** $p < 0.001$ *vs.* control by Dunnett method.

of both COX-2 expression and activity.

DISCUSSION

In the present study, we examined anti-inflammatory effects of TJ-9 in *in vitro* periodontal disease model. We showed that TJ-9 suppressed LPS-induced PGE$_2$ production by HGFs as well as by zymosan-treated monocytes.[6] Therefore, we consider that TJ-9 has anti-inflammatory effects in periodontal disease as well as other inflammatory diseases.

Many various factors such as inflammatory cytokines and chemical mediators are known to be involved in inflammatory responses. In the present study, we examined the productions of IL-6, IL-8 and PGE$_2$ as the index of inflammatory response. IL-6 has an ability to induce osteoclastogenesis[15] and IL-8 acts as a chemoattractant for neutrophils (reviewed in ref. 16). Therefore, these cytokines are closely associated with onset of periodontal disease. In addition, it is widely known that PGE$_2$ leads to inflammatory responses such as vasodilation, enhanced vascular permeability and pain generation. In the present study, we demonstrated that
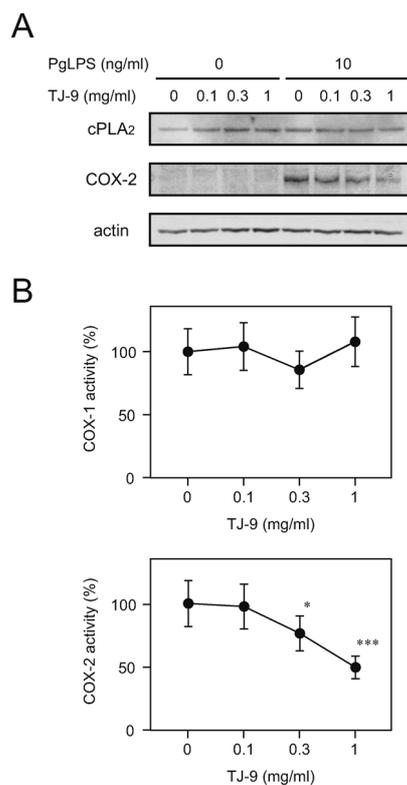
TJ-9 suppresses LPS-induced PGE$_2$ production to basal level (Fig. 2C) but did not affect IL-6 and IL-8 productions (Figs. 2A, B).

PGE$_2$ is produced from phospholipids of cytoplasmic membranes through arachidonic acid cascade. Arachidonic acid released from membrane phospholipids by PLA$_2$ is converted into PGH$_2$ by COX-1 and COX-2, and finally into PGE$_2$. In the present study, TJ-9 suppressed both COX-2 expression and its activity, leading to decreased PGE$_2$ production, while TJ-9 did not alter cPLA$_2$ expression nor inhibit COX-1 activity (Fig. 3). These results reflect the fact that TJ-9 did not affect basal PGE$_2$ production in the absence of LPS (Fig. 2C). From these results, it is suggested that TJ-9 has some components which inhibit COX-2 selectively.

TJ-9 contains several flavonoids such as baicalein, baicalin and wogonin.[17] In particular, baicalein (but not baicalin)[18] and wogonin[19] are reported to suppress COX-2 expression in LPS-treated mouse macrophage. Moreover, wogonin suppresses COX-2 expression in mouse skin fibroblasts treated with tetradeanoyl phorbol acetate (TPA), IL-1β or TNF-α.[20] Therefore, it is suggested that at least baicalein and wogonin suppress COX-2 express in LPS-treated HGFs (Fig. 3A). In addition, TJ-9 includes steroidal compound such as saikosaponins. Saikosaponins have a steroidal structure and show anti-inflammatory effects.[21] However, these steroidal components may be unlikely because, if so, the expression of PLA$_2$ as well as COX-2 may be suppressed.

The reason that TJ-9 suppresses COX-2 expression but not

IL-6 and IL-8 expression may be due to the difference in transcriptional regulation in promoter region. Baicalein inhibits CCAAT/enhancer binding protein β (C/EBPβ) DNA-binding activity in COX-2 promoter region and suppresses COX-2 expression in mouse macrophages,[18] while C/EBPβ plays no or little role in IL-6 and IL-8 transcription in LPS-treated dermal fibroblasts and LPS-treated rheumatoid arthritis synovial fibroblasts.[22]

Taken together, our results suggest that the mechanism of anti-inflammatory effects of TJ-9 is mainly the suppression of PGE$_2$ production rather than those of inflammatory cytokines. Moreover, this decreased PGE$_2$ production will cause substantial reduction of PGE$_2$ in periodontal tissue because periodontal tissue is mainly occupied by HGFs.

Many studies demonstrated that NSAIDs administration prevents gingival inflammation (summarized in ref. 2). And several clinical studies indicated that the concentration of PGE$_2$ in gingival crevicular fluid (GCF) is increased in periodontal disease[23] and is decreased by the administration of NSAIDs such as oral administration with flurbiprofen[24] and mouse wash with ketrolac.[25] Considering the facts that both NSAIDs and TJ-9 suppress PGE$_2$ production, it is possible that administration of TJ-9 also decreases PGE$_2$ concentration in GCF and results in the improvement of gingival inflammation. Therefore, TJ-9 may be useful for the improvement of gingival inflammation in periodontal disease. Importantly, TJ-9 inhibits only COX-2 activity (Fig. 3B), while acid NSAIDs inhibit both COX-1 and COX-2 activities. Because PGE$_2$ produced by COX-1 protects gastric mucosa, these results suggest that TJ-9 may have minimal gastrointestinal dysfunction. Although COX-2 selective inhibitors are used to avoid gastrointestinal dysfunction, recent studies revealed that COX-2 selective inhibitors increase the risk of arthetothrombosis[26] and ischemic stroke.[27] Therefore, TJ-9 may show the similar side effects as COX-2 selective inhibitors. However, the ischemic stroke risk is thought to be by additional pharmacological properties other than COX-2 inhibition because not all COX-2 selective inhibitors show the risk (*e.g.* the risk of rofecoxib is significantly high than control but that of celecoxib is not significant).[27] Moreover, the ability of TJ-9 to selectively inhibit COX-2 may lead to these disorders at very low risk because the occurrence of these disorders by TJ-9 administration has not been reported. Taking into consideration these findings, although the investigation is needed whether TJ-9 has the risk of these disorders, TJ-9 may be useful for the improvement of inflammation in periodontal disease.

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