Abstract. Although a large number of Kampo, Japanese traditional medicines, have been used for the treatment of oral diseases, little is known on their relative potency and endotoxin contamination. In order to obtain basic data for clinical applications, 10 Kampo, and 25 constituent plant extracts were tested for the contamination of lipopolysaccharide (LPS)-like substances, and anti-inflammatory activity. Human gingival (HGF) and periodontal ligament fibroblasts (HPLF) were cultured in 10% fetal bovine serum supplemented with Dulbecco’s modified Eagle’s medium. Viable cell number was measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Prostaglandin (PGE2) was determined by enzyme immunoassay. Cyclo-oxygenase (COX)-1 and COX-2 protein expressions were determined by western blot. COX activity was measured using Cox Inhibitor Screening Assay Kit. LPS, quantified by Endotoxin assay kit, was undetectable or relatively low in the test samples except for rikkosan and unsaiin. Hangeshashinto potently inhibited PGE2 production by interleukin (IL)-1β-stimulated HPLFs and HGFs. Hangeshashinto suppressed the expression of COX-2 protein, but not that of COX-1 protein in IL-1β-induced HGF cells. Hangeshashinto slightly, but not significantly, inhibited both COX-1 and COX-2 activity. The present study provides the basis for clinical application of hangeshashinto for the treatment of stomatitis.

Kampo, Japanese traditional medicines are defined as drugs that are produced by mixing multiple numbers of “seeds, leaves, rhizomes, roots and shells of natural kingdom, and insects” that exhibit medicinal effects under the constant law. Kampo medicine prescriptions were systematically defined through the thousand years’ investigations of the combination effects of many herbal drugs and hazardous effects. The number of species of constituent plant extracts (usually prepared by hot-water extraction or appropriate solvent and lyophilized) presently used in Kampo medicines nearly totals 300. More than 80 reports have investigated the anti-inflammatory effect of Kampo medicines [reviewed in (1) and (2)]. However, basic research and clinical studies for the treatment of oral diseases is much less, including our studies (3-19). Furthermore, little is known on the relative potency of Kampo medicines and their active ingredients. As far as we know, there is no report on the contamination of Kampo medicine by bacterial products derived from the soil such as endotoxin (lipopolysaccharide, LPS).

In the present study, we first investigated LPS contamination in 10 Kampo medicines and 25 constituent plant extracts (supplied as lyophilized materials of hot-water extracts). It was recently reported that orento (7), shosaikoto (8) and hangeshashinto (9) prevented LPS-stimulated inflammation of human gingival fibroblasts (HGFs). However, we found that interleukin (IL)-1β stimulated the prostaglandin (PGE2), IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) production by HGFs to much higher extent than that achieved by LPS prepared from Escherichia coli and Porphyromonas gingivalis (20). Therefore, we investigated whether hangeshashinto also inhibits PGE2 production by IL-1β-stimulated HGFs as well as human periodontal ligament fibroblasts (HPLFs), in order to seek more broader application to oral diseases.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Glycyrrhizin, Wako Pure Chem. Ind. (Osaka, Japan); Dulbecco’s modified Eagle’s medium (DMEM), Invitrogen Corp (Carlsbad, CA, USA); fetal bovine serum (FBS), Gemini Bio-Products (Woodland, CA, USA); 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide.
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We first confirmed that IL-1β significantly enhanced the production of PGE$_2$ in both HGFs and HPLFs (Figure 1B and 2B). Hangeshashinto at a concentration of about 0.0625 mg/ml significantly inhibited cytokotoxic activity towards the reaction buffer (950 μl), and incubated for 2 min at 37°C. Differences were considered significant at $p<0.05$. The results are expressed as means±SD of triplicate experiments. The statistical differences between control and treated groups were evaluated by Student’s $t$-test. Comparison between multiple groups was done by Dunnett’s test. Differences were considered significant at $p<0.05$.

**Assay for cytokotoxic activity.** Cells were treated as described below, and the relative viable cell number was then determined by the MTT method. In brief, the culture medium was replaced with MTT (0.2 mg/ml) dissolved in DMEM, and cells were incubated for 2 h at 37°C. After replacing the medium, the formazan product was dissolved with DMSO, and the absorbance of the lysate at 540 nm was measured. The inhibition of COX activity (%) was measured by the decrease in the absorbance.

**Measurement of endotoxin contamination.** The content of LPS-like substance in the samples was measured using Endotoxic assay kit (ToxinSensor™ Chromogenic LAL; GenScript USA). In brief, the sample was incubated for 30 min at 37°C with the LAL reagent, and the change in the absorbance at 540 nm was measured. Assuming that 1 endotoxin unit (EU) is equivalent to 0.1 ng LPS, LPS contamination in 1 g sample was determined.

**Statistical analysis.** The experimental data are expressed as means±SD of triplicate experiments. The statistical differences between control and treated groups were evaluated by Student’s $t$-test. Comparison between multiple groups was done by Dunnett’s test. Differences were considered significant at $p<0.05$.

**Results**

**Assay for viable cell number.** Hangeshashinto at concentrations up to 2 mg/ml showed no cytotoxicity against HPLF (CC$_{50}$=4.27 mg/ml) (Figure 1A). Hangeshashinto at concentrations up to 1 mg/ml showed no cytotoxicity against HGF cells (CC$_{50}$=5.52 mg/ml) (Figure 2A). Based on these data, the subsequent experiments were carried out with hangeshashinto of 1 or 2 mg/ml for HGFs and HPLFs, respectively.

**Measurement of PGE$_2$ production.** We first confirmed that IL-1β significantly enhanced the production of PGE$_2$ in both HGFs and HPLFs (Figure 1B and 2B). Hangeshashinto at a concentration above 0.0625 mg/ml significantly inhibited...
PGE2 production by IL-1β-stimulated HPLFs, with a 50% inhibitory concentration (IC50) of 0.015 mg/ml. The SI was calculated to be 285 (CC50/IC50=4.27/0.015) (Figure 1B).

Similarly, hangeshashinto above 0.125 mg/ml significantly inhibited PGE2 production by IL-1β-stimulated HGF, with IC50 of 0.055 mg/ml. The SI value was calculated to be 100 (CC50/IC50=5.52/0.055) (Figure 2B).

Measurement of COX-1 and COX-2 protein expression. Hangeshashinto at 1 mg/ml inhibited the expression of IL-1β-induced COX-2 protein, but did not affect the expression of COX-1 protein in HGFs, whereas hangeshashinto affected neither COX-1 nor COX-2 protein expression in HPLFs (Figure 3).

Inhibition of purified COX activity. Hangeshashinto inhibited COX-1 activity slightly but not significantly. Hangeshashinto reduced COX-2 activity by approximately 50%, but also not significantly (Figure 4).

Measurement of endotoxin contamination. Glycyrrhizin (1 g) contained undetectable amount of LPS-like substances (less than 0.7 ng) (estimated by Endotoxin assay kit). Two Kampo medicines (rikkosan and unseiin) contained much higher concentrations of LPS-like substances (>200 ng/g) (Table I). On the other hand, four constituent plant extracts (Alisma rhizome, Scutellaria root, Pinellia tuber, Poria sclerotium) contained undetectable amounts of LPS contamination (below

Figure 1. A: Effect of hangeshashinto on the viability of unstimulated and interleukin (IL)-1β-stimulated human periodontal ligament fibroblasts (HPLFs). HPLFs were incubated for 24 h without (control) or with 5 ng/ml IL-1β in the presence of the indicated concentrations of hangeshashinto, and the relative viable cell number was determined by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide method. B: Concentration-dependent effect of hangeshashinto on prostaglandin (PGE2) production by (IL)-1β-stimulated HPLFs. HPLFs were treated for 24 h with the indicated concentrations of hangeshashinto in the presence or absence of 5 ng/ml IL-1β. The concentration of PGE2 in the medium was then determined. Each value represents the mean±SD of triplicate assays. Comparison between multiple groups used Dunnett's test. *Significant difference at p<0.01.
Another seven Kampo medicines (hotyuekkito, byakkokaninjinto, ninjinyoeito, shosaikoto, juzentaihoto, saireito, kikyoto) and 21 plant constituents (Glycyrrhiza, Cimicifuga rhizome, Japanese Gentian, Asiasarum root, Saposhnikovia root, Coptis rhizome, Phellodendron bark, Polyporus sclerotium, Ginseng, Gardenia fruit, Japanese Angelica root, Platycodon root, Peony root, Astragalus root, Bupleurum root, Jujube, Cnidium rhizome, ginger, Cinnamom bark, Rehmannia root, Atractylodes lancea rhizome) contained 2.1-18.8 ng LPS/g (Table I).

Discussion

The present study demonstrated for the first time to our knowledge that 10 Kampo, Japanese traditional medicine formulations and 25 constitutional plant extracts were contaminated with different amounts of LPS-like substances. Glycyrrhizin, the main component of Glycyrrhiza, and four constituent plant extracts (Alisma rhizome, Scutellaria root, Pinellia tuber, Poria sclerotium) contained undetectable amounts of LPS-like substances (below 0.7 and 2 ng/g.
respectively). Asiasarum root also contained a very low concentration of LPS (2.1 ng/g). Hangeshashinto contained a slightly higher amount of LPS-like substances (less than 8.7 ng/g). Thus, when hangeshashinto was added to culture medium at 10-1000 μg/ml, LPS contamination would be expected to be 0.087-8.7 pg/ml, which would not significantly affect the experimental data. Most other Kampo medicines and constituent plant extracts contained a relatively low concentration of LPS contamination (10.4-18.8 ng/g) (Table I). On the other hand, rikkosan and unseiin contained unexpectedly high concentrations of LPS-like substances (>200 ng/g), which may counteract their anti-inflammatory activity.

We next investigated the anti-inflammatory effect of hangeshashinto in IL-1β-stimulated HGFs and HPLFs, model systems of gingivitis and periodontitis. We previously reported that IL-1β treatment of HGFs resulted in two orders of magnitude increase in COX-1 and COX-2 protein expression. In this study, we found that hangeshashinto inhibited the expression of COX-1 and COX-2 in a dose-dependent manner. The effect was most pronounced at 1 mg/ml, where the protein expression was reduced by 50% compared to the control. These findings suggest that hangeshashinto has potential as a therapeutic agent for inflammatory diseases such as gingivitis and periodontitis.

Table I. Contamination by lipopolysaccharide (LPS)-like substances of Kampo, Japanese traditional medicines (bold) and constituent plant extracts (supplied as lyophilized material of hot-water extracts), and glycyrrhizin (bold).

<table>
<thead>
<tr>
<th>Medicine</th>
<th>LPS (ng/g)</th>
<th>LPS (ng/g)</th>
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<tbody>
<tr>
<td>Hotyuekkito</td>
<td>10.7</td>
<td>Ginseng</td>
</tr>
<tr>
<td>Byakkokaninjinto</td>
<td>16.9</td>
<td>Gardenia fruto</td>
</tr>
<tr>
<td>Ninjinyoeito</td>
<td>18.5</td>
<td>Japanese Angelica root</td>
</tr>
<tr>
<td>Shosaikoto</td>
<td>18.8</td>
<td>Platycodon root</td>
</tr>
<tr>
<td>Juzentaihoto</td>
<td>17.9</td>
<td>Peony root</td>
</tr>
<tr>
<td>Saireito</td>
<td>16.7</td>
<td>Astragalus root</td>
</tr>
<tr>
<td>Kikyoto</td>
<td>17.8</td>
<td>Alisma rhizome</td>
</tr>
<tr>
<td>Unseiin</td>
<td>&gt;200</td>
<td>Bupleurum root</td>
</tr>
<tr>
<td>Rikkosan</td>
<td>&gt;200</td>
<td>Jujube</td>
</tr>
<tr>
<td>Hangeshashinto</td>
<td>8.7</td>
<td>Cnidium rhizome</td>
</tr>
<tr>
<td>Glycyrrhiza</td>
<td>18.8</td>
<td>Ginger</td>
</tr>
<tr>
<td>Cinicifuga rhizome</td>
<td>14.9</td>
<td>Scutellaria root</td>
</tr>
<tr>
<td>Japanese Gentiana</td>
<td>16.2</td>
<td>Cinnmon bark</td>
</tr>
<tr>
<td>Asiasarum root</td>
<td>2.1</td>
<td>Pinellia tuber</td>
</tr>
<tr>
<td>Saposhnikovia root</td>
<td>12.7</td>
<td>Poria sclerotium</td>
</tr>
<tr>
<td>Coptis rhizome</td>
<td>16.3</td>
<td>Rehmannia root</td>
</tr>
<tr>
<td>Phellodendron bark</td>
<td>14.4</td>
<td>Atractylodes lancea rhizome</td>
</tr>
<tr>
<td>Polyporus sclerotium</td>
<td>18.6</td>
<td>Glycyrrhizin</td>
</tr>
<tr>
<td>Glycyrrhizin</td>
<td>&lt;0.7</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean±SD from three independent experiments.
higher production of IL-6, IL-8, MCP-1 and PGE₂, without induction of nitric oxide (NO) and tumor necrosis factor (TNF-α), in contrast to activated macrophages (20). The present study demonstrated for the first time that IL-1β treatment also stimulated HPLFs to produce two-order higher amounts of PGE₂. We found that hageshashinto inhibited PGE₂ production by both IL-1β-stimulated HGFs and HPLFs, at SI values of 100 and 285, respectively. There was a possibility that hageshashinto may have interfered with the coloring reaction of the PGE₂ assay kit. We first confirmed that hageshashinto did not induce PGE₂ production without cells [indicated by cell (--), Figure 2B]. We also found that even when we added hageshashinto to the culture medium of IL-1β-treated cells, the amount of PGE₂ production did not change [indicated by HF+CM in Figure 2B]. These experimental data eliminated this possibility, suggesting that the inhibition by hageshashinto was due not simply to the interference of the PGE₂ assay system. We also found that more than 80% of PGE₂ produced by IL-1β-stimulated cells was found in the medium fraction (data not shown), indicating that IL-1β stimulated the actual production of PGE₂, rather than stimulating its release.

The present study suggests that the inhibition of PGE₂ production by hageshashinto is mainly due to its inhibition of the expression of COX-2 protein, rather than that of COX-1 protein, or of COX-1 and COX-2 activity. This is in agreement with a previous finding that hageshashinto inhibited PGE₂ production via COX-2 protein expression in HGFs and human oral keratinocytes (22). We previously reported that the SI of rikkosan was low (SI=4) (10), possibly due to the higher background level of PGE₂ production by contaminating LPS. It should be noted that the SI value (SI=100) for hageshashinto was 25 times higher than rikkosan, substantiating its efficacy against stomatitis. This may explain why among 150 Kampo medicines, only hageshashinto, ourento and inchinkouto are used for the treatment of stomatitis (23). A single pack (1.875 g) of hageshashinto is administered to adult patients (over 15 years of age) in the clinic. Assuming the bioavailability of hageshashinto to be 100%, its serum concentration is estimated to be 0.47 mg/ml. This concentration is lower than the cytotoxicity concentrations (1 mg/ml), and 9-31-times higher the 50% effective dose (0.015-0.055 mg/ml), which may be sufficient to exert its pharmacological effects.

As far as we know, this is the first report to present SI values for the comparison of relative anti-inflammatory activity against HGFs and HPLFs. A further comparative study using this index may identify the most appropriate plant extracts for the treatment of stomatitis, and elucidate its pharmaceutical action.

In conclusion, the present study demonstrated that most Kampo medicines contain undetectable to relatively low concentrations of LPS-like substances, except for rikkosan and unseiin, and hageshashinto had relatively higher anti-inflammatory activity against both human stomatitis and periodontitis model systems, further substantiating its therapeutic potential.

References


