Anti-inflammatory Potential of Rikkosan Based on IL-1β Network Through Macrophages to Oral Tissue Cells

NORIO HORIE1,2, KEN HASHIMOTO3, SHUNSUKE HINO2, TAKAO KATO2, TETSUO SHIMOYAMA2, TADAYOSHI KANEKO1,4, KAORU KUSAMA1 and HIROSHI SAKAGAMI3

Divisions of 1Pathology and 3Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama, Japan; 2Department of Oral Surgery, Saitama Medical Center, Saitama Medical University, Kawagoe, Saitama, Japan; 4Department of Oral and Maxillofacial Surgery, Nihon University School of Dentistry, Chiyoda-ku, Tokyo, Japan

Abstract. Rikkosan is a traditional Kampo medicine using the control of oral pain caused by dental caries, pulpitis, periodontitis and stomatitis. In order to provide evidence for its clinical effects, we herein investigated whether Rikkosan inhibits the production of pro-inflammatory substances in human and mouse models of inflammation. Rikkosan alone did not induce prostaglandin E2 (PGE2) production, but inhibited interleukin-1β (IL-1β) (5 ng/ml)-stimulated PGE2 production in human gingival fibroblasts and human periodontitis fibroblasts, with a selectivity index higher than 4.0 and 4.3, respectively. Rikkosan alone dose-dependently stimulated tumor necrosis factor-α (TNF-α) production, reaching a peak level slightly lower than that attained by lipopolysaccharide (LPS) at 0.4 mg/ml in mouse macrophage-like RAW264.7 cells. At a higher concentration of Rikkosan (4 mg/ml), TNF-α production, however, declined significantly regardless of the presence or absence of LPS. Rikkosan dose-dependently inhibited IL-1β production by LPS-stimulated RAW264.7 cells, with a selective index of 7.6. Five constituent extracts of Rikkosan, either alone or in combination, showed similar effects on TNF-α and IL-1β productions in activated RAW264.7 cells, but to lower extents than that of Rikkosan. These results demonstrated that Rikkosan inhibited both IL-1β production by LPS-activated macrophages and PGE2 production by IL-1β-stimulated human gingival fibroblasts and human periodontal ligament fibroblasts, suggesting that anti-inflammatory effects of Rikkosan may partially be generated by the inhibition of these pro-inflammatory substances via the IL-1β network through macrophages to oral tissue cells.

Rikkosan is Kampo medicine (Japanese traditional medicines) prescribed for the alleviation of oral pain generated by dental caries, pulpitis, periodontitis, stomatitis and tooth extraction. Rikkosan is a cinnamon color powder with slightly sweet and bitter taste, and is composed of five major constituent herb extracts, Kanzo, Shoma, Ryutan, Saishin and Bofu (Table I). We previously investigated the mechanism of anti-inflammatory effects of Rikkosan using mouse macrophage-like RAW264.7 cells (1-3). In the arachidonic acid (AA) cascade, two enzymes are involved in the production of prostaglandin (PG) E2. PG synthesis begins with the liberation of AA, the prime precursor, from membrane phospholipids by phospholipase (PL) A2. Subsequently, cyclooxygenase (COX) catalyzes the rate-limiting reactions for PG synthesis comprising the bis-cyclooxygenation of AA to form PGG2 and the peroxidative reduction of PGG2 to PGH2 (4). Cytoplasmic PLA2α is activated by pro-inflammatory cytokines or growth factors and catalyzes AA from the cell surface membrane (5, 6). COX has two isoforms, COX-1 (constitutive isoform) and COX-2 (inducible isoform) (7). We reported that low concentrations (0.04-0.4 mg/ml) of Rikkosan stimulated PGE2 production, whereas a higher concentration (4.0 mg/ml) Rikkosan inhibited lipopolysaccharide (LPS)-stimulated PGE2 production, suggesting the presence of both stimulator(s) and inhibitor(s) of PGE2 production in Rikkosan (2). Western blot analysis demonstrated that Rikkosan and all its constituent herb extracts enhanced the intracellular concentration of COX-2 protein, whereas Rikkosan did not significantly affect the expression of PLA2 protein (2, 3). Rikkosan dose-dependently inhibited the COX-2-catalyzed PGE2 production and a higher concentration was required to inhibit the COX-1-catalyzed PG production (3).
Periodontitis is defined as the inflammation of periodontal supporting tissue caused by specific pathogenic microorganisms, resulting in advanced inflammatory infiltration, the destruction of the gingival connective tissue and the periodontal ligament, and alveolar bone loss (5). LPS derived from an oral bacterium such as *Porphyromonas gingivalis* stimulates not only macrophages, but also human gingival fibroblasts to produce pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) and these cytokines damage the periodontal tissue (8-10). PGE₂, likely to be involved in the pathogenesis of periodontal disease, is produced upon stimulation with these cytokines by gingival fibroblasts and smooth muscle cells (11). Among these cytokines, IL-1β was clearly associated with the severity of periodontitis and thereby the level of IL-1β is considered to be a useful diagnostic marker of periodontal diseases (12, 13). Using cultured human gingival fibroblasts (HGFs) and human periodontal ligament fibroblasts (HPLFs), we found that stimulation of these cells by IL-1β produced higher amounts of PGE₂ than when induced by LPS (14, 15). We have reported the anti-inflammatory effect of various herbal medicines such as *Sasa senanensis* Rehder extract on HGFs and HPLFs stimulated with IL-1β (15). There have been no experimental reports to examine the anti-inflammatory effect of Rikkosan on gingivitis and periodontitis. In order to elucidate the possible preventive or therapeutic effect of Rikkosan on inflammatory diseases, we investigated whether Rikkosan inhibits PGE₂ production by IL-1β-stimulated HGFs and HPLFs, and TNF-α and IL-1β production by LPS-stimulated RAW264.7 cells.

**Materials and Methods**

**Materials.** Rikkosan (Lot No. 2990100010) was obtained from Tsumura Corp., Tokyo, Japan. The five ingredients of Rikkosan and their major components are listed in Table I. The following chemicals and reagents were obtained from the indicated companies. Dulbecco’s modified Eagle’s medium (DMEM), phenol red-free DMEM: Invitrogen Corp, Carlsbad, CA, USA; fetal bovine serum (FBS): Gemini Bio-Products, Woodland, CA, USA; dimethyl sulfoxide (DMSO): Wako Pure Chem. Ind., Osaka, Japan; *Escherichia coli* LPS (Serotype 0111:B4) and 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Sigma Chem. Ind., St. Louis, MO, USA; PGE₂ Express enzyme immunoassay (EIA) Kit: Cayman Chemical Co, Ann Arbor, MI, USA; and Mouse IL-1β EIA Kit, TNF-α EIA Kit: R&D Systems, Minneapolis, MN, USA. Culture plastic dishes, 6-well, 24-well and 96-microwell plates were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

**Table I. Five ingredients of Rikkosan (RKS) and their major components.**

<table>
<thead>
<tr>
<th>Constituent herb extract</th>
<th>Major components</th>
<th>Content*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanzo</td>
<td>Glycyrrhizin, glabrid acid, liquiritin, licorice, licoflavone</td>
<td>1.5</td>
</tr>
<tr>
<td>Shoma</td>
<td>Cimigenol, dahurinol, acerinol, β-sitosterol, cimicifugoside</td>
<td>2.0</td>
</tr>
<tr>
<td>Ryutian</td>
<td>Gentiopicroside, trifloroside, swertiamarin, gentisin</td>
<td>1.0</td>
</tr>
<tr>
<td>Saishin</td>
<td>β-Pinene, eucarvone, 1,8-cineole, L-asararin, higenamine</td>
<td>2.0</td>
</tr>
<tr>
<td>Bofu</td>
<td>Deltoin, bergapten, psoralen, hamaudol, cimifugin</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* RKS (7.5g) contains the 1.5g of dried extract composed of five constituent herb extract at the indicated ratio (w/w).

**Assay for viable cell number.** HGFs and HPLFs were established from the periodontal tissues of the first premolar extracted from a mandible, as described previously (16, 17). The life span of HGFs and HPLFs was about 40 population doubling level (PDL), and the cells at 10-20 PDL were used in the present study. HGFs and HPLFs were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate under a humidified atmosphere with 5% CO₂. HGFs and HPLFs were inoculated at 1:3 split ratio in 96-microwell plates, and incubated for 48 h to achieve complete adherence of the cells. Near confluent HGFs and HPLFs were cultured for 24 h in fresh medium containing different concentrations of Rikkosan. The relative viable cell number of the attached cells was determined by MTT method. In brief, the cells were incubated for 1 hour with 0.2 mg/ml MTT. The cells were dissolved in 0.1 ml DMSO and the absorbance at 540 nm of the cell lysate was determined. From the dose-response curve, the 50% cytotoxic concentration (CC₅₀) was calculated.

**Measurement of PGE₂ production.** HGFs and HPLFs were inoculated at 1:3 split ratio in 24-well plates, and incubated for 48 h to achieve complete adherence of the cells. These cells were incubated for 24 h in fresh culture medium containing different concentrations of Rikkosan in the presence or absence of IL-1β (5 ng/ml). The culture supernatant was collected by centrifugation, and the PGE₂ concentration (IC₅₀) for the inhibition of PGE₂ production was calculated and the selective index (SI) was determined by the following equation: SI=CC₅₀/IC₅₀ (18).

**Assay for TNF-α and IL-1β production.** RAW264.7 cells (3×10⁴ cells/well) were inoculated on 96-microwell plate, and incubated for 24 h to achieve complete adherence of the cells. The cells were incubated in fresh phenol red-free DMEM medium supplemented with 10% FBS containing different concentrations of Rikkosan or its components in the presence or absence of LPS (100 ng/ml). For the determination of IL-1β, LPS (1.0 and 10 μg/ml) was used as stimulator. The culture supernatant was collected by centrifugation, and TNF-α and IL-1β concentrations were determined according to the manufacturer’s recommended procedures (1). The limit of
quantitation of these cytokines was 4.8 pg/ml. We confirmed that the culture medium did not contain detectable amounts of IL-1β and TNF-α, and Rikkosan did not affect the colorimetric reaction of EIA for the determination of IL-1β and TNF-α (data not shown).

Statistical analysis. The mean values and standard deviations were calculated. One-way analysis of variance was performed to detect significant effects of the variables. Bonferroni’s multiple comparison test was used at a p-value of 0.05.

Results

Effect on growth and PGE2 production in human oral normal cells. Rikkosan was weakly cytotoxic against both HGFs and HPLFs with and without IL-1β (5 ng/ml) stimulation (HGF: CC50>4 mg/ml without IL-1β, CC50>4 mg/ml with IL1-β; HLPF, CC50=3.078 mg/ml without IL1-β, CC50>4 mg/ml with IL1-β) (Figure 1, Table II). When HGFs and HPLFs were stimulated with IL-1β, PGE2 production and release into the culture medium increased from 0.2 to 1.3 ng/ml, and 0.2 to 3.7 ng/ml, respectively (data not shown). Rikkosan alone did not induce significant PGE2 production in both HGFs and HPLFs cells. Rikkosan inhibited the PGE2 production by IL-1β-stimulated HGFs most efficiently at 1 mg/ml, but the effect was less at higher concentrations (2-4 mg/ml) (Figure 1A). Similarly, Rikkosan inhibited PGE2 production by IL-1β-stimulated HPLFs most efficiently at 2 mg/ml, the effect decreasing at higher concentration (4 mg/ml) (Figure 1B). The SI was >4.0 for HGF and >4.3 for HPLFs (Table II).

Effect on TNF-α production on activated mouse macrophages. Addition of Rikkosan dose-dependently stimulated the production of TNF-α, reaching a peak level
slightly lower than that attained by LPS at 0.4 mg/ml in mouse macrophage-like RAW264.7 cells. At a higher concentration of Rikkosan (4 mg/ml), TNF-α production, however, declined significantly regardless of the presence or absence of LPS (Figure 2A). Similarly, Rikkosan stimulated PGE2 production, reaching a peak level comparable with that attained by LPS, while a higher concentration of Rikkosan inhibited PGE2 production in RAW264.7 cells (2).

In order to investigate the effect of the constituent herb extracts of Rikkosan, the concentration was set to reflect the concentration present in 0.4 mg/ml of Rikkosan. Saishin and Shoma, which stimulated TNF-α production to a comparable extent with that attained by Rikkosan, and inhibited LPS-stimulated TNF-α production to almost the same level with Rikkosan (Figure 2B). On the other hand, Bofu, Ryutan and Kanzo that stimulated the TNF-α production much less, did not affect LPS-stimulated TNF-α production (Figure 2B).

Table II. Inhibition of prostaglandin E2 (PGE2) and interleukin-1β (IL-1β) production by Rikkosan in human gingival (HGF) and periodontal ligament (HPLF) fibroblasts, cells and mouse macrophage-like cells (RAW264.7).

<table>
<thead>
<tr>
<th>Cell</th>
<th>IL-1β</th>
<th>CC50 (mg/ml)</th>
<th>IC50 (mg/ml)</th>
<th>SI=CC50/IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF</td>
<td>With</td>
<td>&gt;4</td>
<td>0.99 (PGE2 production)</td>
<td>&gt;4.0</td>
</tr>
<tr>
<td></td>
<td>Without</td>
<td>&gt;4</td>
<td>0.94 (PGE2 production)</td>
<td>&gt;4.3</td>
</tr>
<tr>
<td>HPLF</td>
<td>With</td>
<td>3.078</td>
<td>0.518 (IL-1β production)</td>
<td>7.6</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>With</td>
<td>3.915</td>
<td>0.518 (IL-1β production)</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>Without</td>
<td>&gt;4</td>
<td>0.518 (IL-1β production)</td>
<td>7.6</td>
</tr>
</tbody>
</table>

The 50% cytotoxic concentration (CC50) values against RAW264.7 cells are cited from our previous report (1). IC50, the concentration that inhibited the PGE2 or IL-1β by 50%.

Discussion

Recent reports have suggested the possible efficacy of natural products including herbal medicines for periodontal disease (8, 19). Chitosan, cranberry, Scutellaria baicalensis, shosaikoto, orenoto and Sasa senanensis Rehder have been investigated for their anti-inflammatory activity against HGF (15, 20-24). The present study demonstrated that relatively low concentrations (1.0-2.0 mg/ml) of Rikkosan inhibited IL-1β-stimulated PGE2 production in HGFs and HPLFs significantly, and more potently than at 4 mg/ml, suggesting the presence of both stimulator(s) and inhibitor(s) of PGE2 production in Rikkosan. The concentrations of Rikkosan needed for maximum inhibition of PGE2 production were different between HGFs and HPLFs, however, were relatively low, it is important to determine the most appropriate concentrations of Rikkosan for its clinical application.

The present study demonstrated that Rikkosan alone did not induce production of IL-1β in RAW264.7 cells, and inhibited IL-1β production in LPS-stimulated RAW264.7 cells. On the other hand, Rikkosan stimulated the production of TNF-α to a level comparable to that attained by LPS in RAW264.7 cells, and inhibited the stimulation of TNF-α production only at the highest concentration (4 mg/ml). This finding suggests that the mechanism of induction of IL-1β and that of TNF-α differ. Yucel-Lindberg et al. reported that both IL-1β and TNF-α stimulated HGFs to produce the PGE2, but that HGFs stimulated with IL-1β produced higher amounts of PGE2 than those stimulated with TNF-α (14). The inhibition of IL-1β in HGFs and HPLFs may thus be a crucial part of the anti-inflammatory effect of Rikkosan.
The studies of five constituent herb extracts of Rikkosan demonstrated that the inhibition of LPS-stimulated IL-1β production in RAW264.7 cells varied considerably between the herb samples. The combination of Kanzo, Shoma and Saishin inhibited LPS-stimulated IL-1β production to an extent comparable to that of Rikkosan and there was no inhibitory effect of Bofu and Ryutan. As for TNF-α production, the combination of Saishin and Shoma led to a relatively strong inhibition of LPS-stimulated TNF-α production but interestingly, Saishin and Shoma also induced the production of the highest amount of TNF-α. Saishin and Shoma may have both maximum inhibitory and inducing effects on TNF-α production. These results suggest the presence of both stimulator(s) and inhibitor(s) of cytokine production in the samples of Saishin and Shoma.

The mechanism of inhibition of IL-1β-stimulated PGE2 production may be due to the inactivation of NF-κB (27). However, a possible association between the stimulatory effect of IL-1β and the components of Rikkosan has not been studied. Glycyrrhizin, one of the major components of Kanzo, attenuated TNF-α and IL-1β production by the inhibition of NF-κB, and signal transducer and activator transcription-3 activation, in the lung injury mouse model (28). Bofu inhibited TNF-α and IL-1β expression in LPS-stimulated RAW264.7 cells via suppression of NF-κB- and mitogen-activated protein kinases-dependent pathways (29). β-Sitosterol, one of the major components of Shoma, inhibited the expression of IL-1β and TNF-α by inhibiting the NF-κB pathway in a mouse model of 2,4,6-trinitrobenzene sulfonic acid-induced colitis (30). Further studies are required to elucidate the precise molecular anti-inflammatory mechanisms of Rikkosan and its ingredients.
In conclusion, the present study provides evidence for the anti-inflammatory potential of Rikkosan against HGFs and HPLFs. Rikkosan is known to exert an analgesic activity against oral pain. The therapeutic or preventive effects of Rikkosan on oral diseases such as periodontitis and oral mucositis may not be exerted by a simple mechanism, but rather by more complicated mechanisms including analgesic and anti-inflammatory effects. Taken together, anti-inflammatory effects of Rikkosan may partially be generated by the inhibition of these pro-inflammatory substances via the IL-1β network, through macrophage to oral tissue cells.

References


Received January 30, 2014
Revised February 28, 2014
Accepted March 4, 2014