Multi-Omics Analysis of Anti-Inflammatory Action of Alkaline Extract of the Leaves of Sasa sp.

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Abstract: Efficient utilization of alkaline extracts of several plants for the treatment of oral diseases has been reported. To investigate the mechanism of anti-inflammatory activity of alkaline extract of the leaves of Sasa sp. (SE), multi-omics analysis using metabolomics and DNA array was performed. Human gingival fibroblasts (HGFs) were treated for IL-1β to induce inflammation (detected by PGE₂ production in culture medium) in the presence or absence of SE. Both IL-1β and SE showed slight hormetic growth stimulation against HGF. SE inhibited PGE₂ production dose- and time-dependently. Its inhibitory action was more pronounced by first treating the cells with SE, rather than with IL-1β. At 3 h after IL-1β treatment, 18 amino acids (except cysteine and glutamic acid), total glutathione (GSH, GSSG, Cys-GSH disulfide), Met-sulfoxide, 5-oxoproline, and SAM declined, whereas DNA expressions of AKT, CASP3, and CXCL3 were elevated. These changes were reversed by simultaneous treatment with SE. The present study suggests that the anti-inflammatory action of SE is mediated via various metabolic pathways for cell survival, apoptosis, and leukocyte recruitment.

Keywords: alkaline extract of the leaves of Sasa sp.; human gingival fibroblast; IL-1β; anti-inflammation; PGE₂; metabolomics; DNA array; cell survival; apoptosis

1. Introduction

Alkaline extracts of many plants such as licorice root [1], green tea leaf, oolong tea leaf and orange flower [2] have been reported to show much higher anti-HIV activity than water extracts of counterpart plants, possibly due to higher amount of lignin–carbohydrate complexes, which are more efficiently extracted by alkaline solution than hot water. In fact, the anti-HIV activity of the alkaline extract of bamboo leaf declined by removal of the lignin–carbohydrate complex [3]. Although many papers have reported the utilization of alkaline extract for the treatment of oral disease, mechanistic analysis of biological actions such as bone regeneration and anti-inflammation [4], periodontal inflammation and alveolar bone loss [5], anti-oral bacterial activity [6], and alleviation of oral lichenoid dysplasia [7] by alkaline extract has been limited.

Alkaline extract of the leaves of Sasa sp. (SE) showed potent anti-viral activity, and its chemotherapeutic index (safety margin) (SI) (determined by the ratio of CC₅₀ (50% cytotoxic concentration in uninfected cells)/EC₅₀ (50% protective concentration of HIV-infected cells)) was 86, comparable with that of lignin–carbohydrate complex (SI = 12~311 (mean: 76) (5)).
and higher than those of hundreds of chemically defined tannins (SI = 1.1–7.3) (n = 75) and flavonoids (SI = 1.5–24) (n = 114) [8]. Furthermore, SE showed potent anti-inflammation activity (assessed by inhibition of PGE$_2$ production and COX-2 protein expression [8] and interleukin (IL)-8 production [6]) against an IL-1β-induced inflammation model with human gingival fibroblasts (HGFs) [9]. Although many researchers have isolated and determined the chemical structures of thousands of low molecular weight compounds, such as tannins and flavonoids, from the natural kingdom, the yield of purified materials is in the milligram order. In contrast, SE can be obtained in the gram order, and is thus suitable for manufacturing over-the-counter (OTC) drugs such as oral rinse and toothpaste [10]. Upon high-performance liquid chromatography (Develosil RPAQUEOUS (C-30), SE was eluted as a single peak at the retention time of 22.175 min, suggesting that SE is present in a multi-biological complex [11]. Among nearly 30 papers on the anti-inflammatory activity of bamboo extracts, only one paper other than our papers used the alkaline extract [12]. All of them focused on the later stage of inflammation, and none of them considered the dental application.

To clarify the action point of SE, optimal condition for induction of inflammation by IL-1β was first investigated. Since inflammation starts as early as 3 h after IL-1β stimulation, the intracellular metabolites and genes that are upregulated by IL-1β and then downregulated by SE at the earlier stage of inflammation of HGF were identified by metabolomics and DNA microarray analyses, respectively. The earlier stage of inflammation was chosen for the analysis, since at the later stage many different signals may exert non-specific biological actions.

2. Experimental Section

2.1. Materials

The following chemicals and reagents were purchased from the indicated companies: Dulbecco’s modified Eagle’s medium (DMEM) from GIBCO BRL (Grand Island, NY, USA); fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich Inc. (St. Louis, MO, USA); dimethyl sulfoxide (DMSO) from Wako Pure Chem. Ind. (Osaka, Japan); 96-microwell plate from TPP (Techno Plastic Products AG, Trasadingen, Switzerland); a 10 cm dish from Becton Dickinson Labware (Franklin Lakes, NJ, USA); interleukin (IL)-1β, R&D systems (Minneapolis, MN, USA).

SE was prepared by iron ion substitution, alkaline extraction, and neutralization/desalting. Lyophilization and measurement of the dry weight of SE showed that it contained 58.2 ± 0.96 mg solid materials/mL [10,13].

2.2. Cell Culture

Human gingival fibroblasts (HGFs), human periodontal ligament fibroblasts (HPLFs), and human pulp cells (HPCs) were obtained from the first premolar extracted tooth in the lower jaw and periodontal tissues of a twelve-year-old girl, according to the guideline of the Institutional Board of Meikai University Ethics Committee (No. A0808) after obtaining informed consent from the patients [14]. For the present study, cells at the population doubling level (PDL) of 10–15 were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin G, and 100 µg/mL streptomycin sulfate under humidified 5% CO$_2$ atmosphere.

2.3. Assay for Cytotoxic Activity

Cells were seeded at $2 \times 10^3$ cells/0.1 mL in the inner 60 wells of a 96-microwell plate (Falcon Becton Dickinson, Franklin Lakes, NJ, USA). The surrounding 36 exterior wells were filled with 0.1 mL of sterilized water to minimize the evaporation of water from the culture medium. After 48 h, the medium was removed by suction with an aspirator, and replaced with 0.1 mL of fresh medium containing different concentrations of the sample. Cells were incubated for the indicated times, and the relative viable cell number was then determined by the MTT method [9]. Briefly, the treated cells were incubated for another
2 h in a fresh culture medium containing 0.2 mg/mL MTT. Cells were then lysed with 0.1 mL of DMSO and the absorbance at 560 nm of the cell lysate was determined using a microplate reader (Infinite F 50 R, TECAN, Kawasaki, Japan).

2.4. Assay for Pro-Inflammatory Substances

The concentration of PGE\textsubscript{2} released into the culture medium was determined by enzyme immunoassay (EIA) (Cayman Chemical Co., Ann Arbor, MI, USA).

2.5. Processing for Metabolomic Analysis

Near confluent cells in a 10 cm dish were treated for 3 h with 0 (control), IL-1\textbeta (3 ng/mL), SE (1%) or IL-1\textbeta (3 ng/mL) + SE (1%). Aliquots of the cells were trypsinized and the viable cell number was counted with a hemocytometer after staining with trypan blue. The remaining cells were washed twice with 5 mL of ice-cold 5% D-mannitol and then immersed for 10 min in 1 mL of methanol containing internal standards (25 \mu M each of methionine sulfone, 2-[\text{N-morpholino}]ethanesulfonic acid and D-camphor-10-sulfonic acid). The methanol extract (supernatant) was collected. The aqueous layer was filtered to remove large molecules by centrifugation through a 5 kDa cut-off filter (Millipore, Billerica, MA, USA) at 9100 \times g for 2.5 h at 4 °C. The 320 \mu L of the filtrate was concentrated by centrifugation and dissolved in 50 \mu L of Milli-Q water containing reference compounds (200 \mu M each of 3-aminopyrrolidine and trimesate) immediately before capillary electrophoresis-time-of-flight-mass spectrometry (CE-TOF-MS) analysis. The parameters of the measurement instrument and data processing have already been described. The concentrations of intracellular metabolites were expressed as amol/cell [15,16].

2.6. DNA Microarray Processing

Total RNA was extracted from the cells using RNA iso plus reagent (Takara Bio, Tokyo, Japan) according to the manufacturer’s instructions. RNA quantity and quality were determined using the Nano Drop 2000 (Thermo Fisher Scientific K. K., Tokyo, Japan) and RNA 6000 Nano Assay kit on an Agilent 2100 BioAnalyzer (Agilent Technologies Japan, Ltd., Tokyo, Japan), as recommended. RNA samples were used for cRNA target preparation only when the ratio A260:A280 was 1.8–2.1 and the RNA Integrity Number (RIN) value was >7.8.

DNA microarray was performed on the duplicated samples of 4 treatment groups. The Sure-Print G3 Human GE 8 × 60 K Ver. 3.0 array kit (Agilent Technologies Japan, Tokyo, Japan) was used for cRNA target preparation and DNA microarray hybridization detection. Following an assessment of total RNA concentration and quality, 200 ng of the RNA sample from each cell was used for microarray experiments. RNA Spike-In one-color kit (Agilent Technologies Japan, Tokyo, Japan) was used to efficiently monitor their microarray workflow for accuracy. Synthesis of first-strand and second-strand cDNA was performed with T7 promoter primer. Cy3-labeled cRNA was amplified with T7 RNA polymerase Blend and cyanine3-CTP at 40 °C for 2 h and purified using the RNAeasy kit (Qiagen, K.K., Tokyo, Japan). The resulting purified Cy3-labeled cRNA was assessed for concentration, purity, and quantity. In total, 600 ng of Cy3-labeled cRNA was fragmented at 60 °C for 30 min with the fragmentation buffer of Gene Expression Hybridization Kit (Agilent, Tokyo, Japan). The fragmented cRNA with 2× GE Hybridization buffer HI-RPM was loaded into the array slide and hybridized at 65 °C for 17 h. The microarrays were washed with Gene Expression Wash Buffer 1 (Agilent, Tokyo, Japan) at room temperature for 1 min and with Wash Buffer 2 (Agilent, Tokyo, Japan) at 37 °C for 1 min. After drying, the microarrays were immediately scanned with a DNA Microarray Scanner (Agilent, Tokyo, Japan) at 3 \mu m.

Initial data analysis was performed using Feature Extraction software (Agilent, Tokyo, Japan) to exclude outlier pixels, accurately determine feature intensities and ratios, and calculate statistical confidences. Raw intensities were normalized using a 75th percentile
global normalization and law signal cut-off for each spot before further analysis of the quantified data using Subio Platform Basic Plug-in (Subio Inc., Aichi, Japan).

Further information concerning gene function, functional annotation clustering, and the biological and molecular function of each gene analyzed was obtained with Subio Platform Advanced Plug-in (Subio Inc., Aichi, Japan) and KeyMolnet Lite (IMMD Inc., Tokyo, Japan) software.

2.7. Statistical Analysis

Statistical analyses were performed using the Origin Pro 2018 software (Origin Lab Corporation, MA, USA). Experimental data are presented as the mean ± standard deviation (SD) of triplicate determinations. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test for multiple comparisons. A value of p < 0.05 was considered to indicate statistically significant differences.

3. Results

3.1. Pretreatment, Rather Than Post-Treatment, with SE More Efficiently Inhibited IL-1β-Stimulated PGE2 Production in HGF.

3.1.1. Optimal Conditions for Induction of Inflammation by IL-1β

We first set up the optimal condition for measuring the anti-inflammatory action of SE (Figure 1). Interleukin (IL)-1β induced an approximately 100-fold increase in PGE2 production in human gingival fibroblasts (HGFs) and human periodontal fibroblasts (HPLFs), but not in human pulp cells (HPCs) (A). The optimal concentration of IL-1β was 3.1–12.5 (ng/mL). Since higher PGE2 production was observed in HGF than HPLF, we used HGF in the following experiments. The production of PGE2 was observed 3 h after IL-1β (3 ng/mL) treatment and increased up to 48 h (B). The addition of SE up to 10% did not interfere with the actual determination of PGE2 with ELISA (C). Unless otherwise stated, cells were treated for 48 h with IL-1β (3 ng/mL).

![Figure 1](image_url)

Figure 1. Kinetics of inhibition of IL-1β-induced human gingival fibroblast PGE2 production by SE. (A) Dose–response: IL-1β stimulated the production of PGE2 in HGF, HPLF, but not in HPC. (B) Time-course of PGE2 production after IL-1β (3 mg/mL) addition. (C) SE did not interfere with the PGE2 measurement. (D) SE inhibited the IL-1β-stimulated PGE2 production more effectively when HGF cells were first treated by SE before IL-1β addition. (E) Prolonged pretreatment with IL-1β (3 mg/mL) reduced the inhibitory effect of SE to a greater extent.
3.1.2. Inhibition of IL-1β-Induced Inflammation by SE

SE dose-dependently reduced the IL-1β-induced PGE$_2$ production. In the presence of 1 or 2% SE, PGE$_2$ production was reduced to 9.1 and 3.0% of control, respectively. The inhibitory effect of SE was more pronounced when SE was added before IL-1β addition (D). When HGF cells were preincubated for longer times with IL-1β before the addition of SE, the inhibitory effect of SE was progressively reduced (E). However, approximately 25% of PGE$_2$ production remained even if HGF cells were incubated for 5 h with 2% SE (E). This suggests that some parts of SE may bind to IL-1β extracellularly and block its binding to a cellular receptor and then inhibit the signal transduction of IL-1β.

3.1.3. Mild Growth Stimulation Effect of IL-1β and SE

A careful inspection revealed that both IL-1β and SE slightly stimulated the growth of HGF (A, D). Reproduced results were obtained by repeated experiments. This is consistent with our finding that SE stimulates the growth of differentiated rat PC12 neuronal cells in a serum-free medium [17].

3.2. Metabolome Analysis

A total of 153 metabolites were detected. p-Coumaric acid, one of the precursors of the lignin–carbohydrate complex (LCC) [18,19], was detected in both cells and medium, indicating that SE contains LCC as major anti-viral and anti-inflammatory substances. There were many metabolites whose expressions were increased or decreased by IL1-β or by SE. Interestingly, when column 2 and column 3 were compared (Figure 2A), their patterns of ups and downs were found to not be overlapped with each other.

![Figure 2](image-url)

**Figure 2.** Metabolomic analysis of HGF cells incubated for 3 h without (control) (column 1) or with SE (2%) (column 2), IL-1β (3 ng/mL) (column 3) and SE (2%) + IL-1β (3 ng/mL) (column 4). The averaged concentrations of samples ($n = 4$) for each condition are visualized. To determine the color, the average concentration was calculated for each metabolite, and individual metabolite concentration was divided by the average. The metabolites of the cultured cell (A) were aligned by the clustering with Pearson Correlation, and the metabolites of the supernatant (B) were aligned according to the data in (A). The numbers of cells recovered per dish are 1.06 ± 0.086 × 10$^6$, 1.16 ± 0.068 × 10$^6$, 1.21 ± 0.12 × 10$^6$, and 1.12 ± 0.053 × 10$^6$, respectively. Blue, white, and red indicate fold changes of 0.8, 1, and 1.2, respectively, compared to the average of each metabolite. The gray indicates that the corresponding metabolite is not detected. The names of metabolites can be visible by 4-fold magnification with zooming. Original data are available in Supplemental Table S1.
Treatment of HGF cells with IL-1β resulted in a 38% decline of a total of 18 amino acids (except cysteine and glutamic acid) (Table 1). Cysteine was not detected due to the degradation during sample preparation. Additionally, total glutathione (GSH, GSSG, and cysteine-glutathione disulfide), Met-sulfoxide, 5-oxoproline, and S-adenosylmethionine (SAM) declined. These changes were reversed by simultaneous treatment with SE. ATP utilization (assessed by the ratio of AMP/ATP and ADP/ATP) and GTP utilization (assessed by the ratio of GMP/GTP and GDP/GTP) were elevated by 60–88% and 35–44%, respectively, due to IL-1β and were reversed to near the control level by SE (Table 1).

Table 1. Metabolites downregulated by IL-1β and reversed by SE.

| Metabolites (Amol/Cell) | Control (%) | IL-1β (%) | SE (%) | SE + IL-1β (%) |
|-------------------------|-------------|-----------|--------|---------------|
| **Amino acids**         |             |           |        |               |
| Gly                     | 211,177     | 100       | 146,168| 69            |
| Ala                     | 60,888      | 100       | 33,272 | 55            |
| Arg                     | 9902        | 100       | 6464   | 83            |
| Asp                     | 26,445      | 100       | 17,409 | 66            |
| Asn                     | 15,192      | 100       | 8635   | 57            |
| Glu                     | 337,154     | 100       | 197,771| 59            |
| His                     | 287,270     | 100       | 258,709| 90            |
| Ile                     | 13,301      | 100       | 7407   | 56            |
| Leu                     | 35,810      | 100       | 22,531 | 63            |
| Lys                     | 29,974      | 100       | 15,917 | 64            |
| Met                     | 15,489      | 100       | 7274   | 47            |
| Phe                     | 29,015      | 100       | 16,943 | 58            |
| Pro                     | 62,148      | 100       | 94,904 | 80            |
| Ser                     | 47,587      | 100       | 29,061 | 61            |
| Thr                     | 140,117     | 100       | 73,931 | 53            |
| Trp                     | 6312        | 100       | 3447   | 55            |
| Tyr                     | 30,049      | 100       | 17,177 | 57            |
| Val                     | 29,763      | 100       | 19,547 | 66            |
| **Total**               | 1,420,101   | 100       | 954,178| 62            |
| **Glutathiones**        |             |           |        |               |
| GSH                     | 41,743      | 100       | 45,315 | 109           |
| GSSG                    | 19,334      | 100       | 6905   | 36            |
| Cys-GSH disulfide       | 461         | 100       | 27     | 6             |
| **Total**               | 61,538      | 100       | 52,248 | 85            |
| **Others**              |             |           |        |               |
| Met-sulfoxide           | 283         | 100       | 175    | 41            |
| 5-Oxoproline            | 8806        | 100       | 7801   | 89            |
| SAM+                    | 713         | 100       | 324    | 45            |
| **ATP/GTP utilization** |             |           |        |               |
| ATP                     | 64,581      | 100       | 46,778 | 72            |
| ADP                     | 4074        | 100       | 4734   | 116           |
| AMP                     | 425         | 100       | 581    | 136           |
| AMP/ATP                 | 0.0066      | 100       | 0.0124 | 188           |
| ADP/ATP                 | 0.0631      | 100       | 0.1012 | 160           |
| GTP                     | 14,959      | 100       | 12,730 | 85            |
| GDP                     | 747         | 100       | 861    | 115           |
| GMP                     | 162         | 100       | 198    | 123           |
| GMP/GTP                 | 0.0108      | 100       | 0.0156 | 144           |
| GDP/GTP                 | 0.0499      | 100       | 0.0676 | 135           |

Each value is the mean of 4 determinations. SD values of each metabolite are available in Supplemental Table S2.
3.3. DNA Array Analysis

The flow chart of DNA array analysis is shown in Figure 3. After removing inappropriate spots, 57,432 spots were selected. After normalization, the spots within the range of log 2 (2), compared with control, were removed, and the remaining 16,405 spots were subjected to DNA microarray analysis. Next, we extracted the genes whose expressions were enhanced more than twice by IL-1β and reduced to 1/2 by simultaneous addition by SE to obtain 366 genes. These genes were subjected to assay for the KEGG pathway.

![Flow chart of DNA array analysis.](image)

**Figure 3.** Flow chart of DNA array analysis.

Changes in the expression intensity of 16,405 genes are shown in Figure 4. There were many genes whose expressions were increased by IL1-β compared to the control, and there was a group of genes in which the increase by IL1-β was suppressed by SE + IL1-β (Figure 4).

![The expression intensity of each sample was expressed in a logarithmic scale using a line graph as a reference for control. In total, 16,405 spots with fluctuations of Log 2 (2) or more compared to the control were analyzed. Genes with higher mean expression intensity compared to the control are shown in red, and genes with lower mean expression intensity are shown in blue.](image)

**Figure 4.** The expression intensity of each sample was expressed in a logarithmic scale using a line graph as a reference for control. In total, 16,405 spots with fluctuations of Log 2 (2) or more compared to the control were analyzed. Genes with higher mean expression intensity compared to the control are shown in red, and genes with lower mean expression intensity are shown in blue.
IL1-β induced mRNA expression of 26 genes including tumor necrosis factor superfamily member 2 (TNFA) (2.1439-fold), RAC serine/threonine-protein kinase (AKT) (2.32-fold), caspase 3 (CASP3) (1.027-fold). These expression levels were suppressed by adding SE at the same time. In particular, CASP3 was induced about twice as much as the control (2.1072-fold) by adding IL1-β, but it was returned to the control level by adding SE at the same time (Table 2). On the other hand, chemokines such as CXC motif chemokine on the TNF pathway (CXCL3) were increased to a greater extent (2.8826-fold), but their expression did not return to the control level. Highly expressed mRNAs are depicted in Figure 5, where magnitudes of amplification are indicated by the thickness of red circles.

Table 2. Metabolites downregulated by IL-1β and reversed by SE.

| Metabolite | Control | SE | IL-1β | SE + IL-1β |
|------------|---------|----|-------|------------|
| Btk        | 0.000   | 0.000 | 1.108 | 0.000      |
| calpain    | 0.000   | 0.000 | 2.139 | 0.000      |
| calpain 13 | 0.000   | 0.000 | 2.139 | 0.000      |
| collagenase-IV | 0.000 | 0.000 | 3.232 | 0.000      |
| Lefty      | 0.000   | 0.000 | 3.521 | 0.000      |
| Lefty2     | 0.000   | 0.000 | 3.521 | 0.000      |
| MMP        | 0.000   | 0.000 | 3.232 | 0.000      |
| MMP-2      | 0.000   | 0.000 | 3.232 | 0.000      |
| Nbeta      | 0.000   | 0.000 | 1.121 | 0.000      |
| NEX/T      | 0.000   | 0.000 | 1.121 | 0.000      |
| NICD       | 0.000   | 0.000 | 1.121 | 0.000      |
| Notch      | 0.000   | 0.000 | 1.121 | 0.000      |
| Notch2     | 0.000   | 0.000 | 1.121 | 0.000      |
| NRPTP      | 0.000   | 0.000 | 2.017 | 0.000      |
| PKC        | 0.000   | 0.000 | 1.676 | 0.000      |
| PKCd       | 0.000   | 0.000 | 1.676 | 0.000      |
| Shp1       | 0.000   | 0.000 | 2.017 | 0.000      |
| TGFb       | 0.000   | 0.000 | 3.521 | 0.000      |
| TLR        | 0.000   | 0.000 | 2.478 | 0.000      |
| TLR9       | 0.000   | 0.000 | 2.478 | 0.000      |
| TNF        | 0.000   | 0.000 | 1.439 | 0.255      |
| TNFa       | 0.000   | 0.000 | 1.439 | 0.255      |
| AKT        | 0.000   | −0.189 | 2.320 | −0.309     |
| AKT2       | 0.000   | −0.189 | 2.320 | −0.309     |
| caspase    | 0.000   | −0.705 | 1.072 | −0.151     |
| caspase-3  | 0.000   | −0.705 | 1.072 | −0.151     |
| CXCL3      | 0.000   | 0.110 | 8.826 | 7.756      |
| CXCL5      | 0.000   | −0.301 | 3.038 | 1.990      |
| CXCL10     | 0.000   | 0.000 | 3.254 | 2.252      |
| CCL7       | 0.000   | 0.000 | 4.148 | 3.141      |

Data for a total of 366 gene expressions are available in Supplementary Table S3.
4. Discussion
The present study demonstrated that SE inhibited the IL1-β-induced inflammation (PGE2 production and other inflammatory cytokines) [9], and the anti-inflammatory action of SE was partially inhibited when HGF cells were treated with SE after pretreatment with IL1-β (Figure 1). This suggests that one of the anti-inflammatory actions of SE may be mediated by the inhibition of IL1-β binding to its cellular receptor (IL1-βR) by masking the ligand or receptor. Since HGF expresses various cell surface receptors of various inflammatory cytokines upon stimulation [17], SE may non-specifically inhibit the ligand–receptor interactions of all cytokines. Further studies are necessary to test this possibility.

SE contains numerous components including the lignin–carbohydrate complex and its degradation products [13]. Such components bind with each other, producing the aggregated form [11]. At present, it is not clear which components or aggregate formations are responsible for the anti-inflammatory action of SE. The present metabolomic analysis demonstrated that (i) SE contains p-coumaric acid, one of the degradation products of lignin–carbohydrate complex, (ii) it was incorporated into the cells (332 atom/cell), and (iii) IL1-β treatment slightly increased its intracellular concentration (511 amol/cell) with the reduction in the extracellular concentration from 146 to 139 μM (Supplementary Table S4). This suggests the possibility that the anti-inflammatory activity of SE might be in part to p-CA. However, this possibility was eliminated by our finding that the anti-inflammatory activity of p-coumaric acid against HPLF (assessed by chemotherapeutic index CI (=CC50/IC50)) was much less than SE (chemotherapeutic index >3.1 vs. >96.3) (Supplementary Figure S1). We also found that the anti-inflammatory activity of other popular polyphenols such as curcumin, gallic acid, and ferulic acid was very low (CI = 0.94–2.9). On the other hand, Japanese traditional medicines (Kampo), such as Hangeshashinto [20,21] inhibited IL-1β-stimulated PGE2 production, to the comparable extent with that of SE (CI = 100). Endotoxin contamination of Hangeshashinto and SE was 8.7 [22] and <2 ng/g (under the detection limit, possibly due to the cleavage of the
ester bond of LPS during alkaline extraction), respectively. These data indicate that the anti-inflammatory activity of SE is higher than popular low molecular weight polyphenols and comparable with that of Kampo medicines.

The present study demonstrated that IL1-β reduced approximately 40% of intracellular concentrations of amino acids, which was reversed to some extent by the addition of SE (Table 3). At present, the biological significance of this finding is not clear. Similarly, SE rescued the IL1-β-induced decline of total glutathione and Met-sulfoxide and 5-oxoproline to some extent. This is consistent with our previous finding that Met-sulfoxide and 5-oxoproline declined during the aggravation of IL1-β-induced HGF inflammation by TiO$_2$ nanoparticles (Supplementary Figure S2A,B, cited from [15]). Since Met-sulfoxide [23–25] and 5-oxoproline [26–28] are well-known markers reflecting oxidative stress, SE might have induced the cells to fight against oxidative stress.

Table 3. Summary of anti-inflammatory action of SE against IL-1β-treated HGF cells.

| Metabolomic analysis                        | + IL-1β | +SE+ IL-1β |
|---------------------------------------------|---------|------------|
| 19 Amino acids (except Cys and Glu)         | ↓       | →          | ↑          |
| Total glutathione (GSH, GSSG, Cys-GSH disulfide) | ↓       | →          | ↑          |
| Met-sulfoxide                               | ↓       | →          | ↑          |
| 5-Oxoproline                                | ↓       | →          | ↑          |
| SAM                                         | ↓       | →          | ↑          |

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| AKT (Cell survival)                         | ↑        | →          | ↓          |
| CASP3 (Apoptosis)                           | ↑        | →          | ↓          |
| CXCL3 (Leukocyte recruitment)               | ↑        | →          | ↓          |

We have previously reported that SAM level step-wisely declined with aggravation of inflammation triggered by IL1-β then TiO$_2$ nanoparticles (Supplementary Figure S2C). The present study showed that, during recovery from inflammation by SE, SAM returned to near the control level (Table 2). SAM is synthesized in a cyclic pathway (termed one-carbon metabolism) from methionine and ATP by methionine adenosyltransferase (EC 2.5.1.6). During inflammation, methionine and ATP levels decline, thus leading to the decline of SAM, which is a well-known methyl group transfer. Since methylation of DNA and histone is known to repress the expression of various genes, a poor supply of SAM may lead to the enhanced expression of pro-inflammatory cytokines [29]. Therefore, reversal of SAM by SE can be considered to be the trigger of anti-inflammatory action.

IL-1β is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The present DNA microarray analysis demonstrated the activation of four independent pathways by IL1-β, which were also reversed by SE: tumor necrosis factor superfamily member 2 (TNFA), RAC serine/threonine-protein kinase (AKT), and caspase 3 (CASP3) (Figure 5, Table 3). An increase in the ATP and GTP utilization favors the commitment of cells towards apoptosis since the elevation of cytosolic ATP level is a requisite to the apoptotic cell death process [30]. Several papers have reported the mild apoptosis induction (detected by annexin staining) of HGF by IL-1β [31]. IL-1β also induced mild growth stimulation of HGF by upregulation of NF-κB pathway members [32], supporting our data. IL-1β also increased matrix metalloproteinase (MMP) levels [33,34]. Simultaneous stimulation with transforming growth factor (TGF)-β1 or IL-1β induce weak apoptosis and then augment IL-8 and VEGF production [35]. Such weak apoptosis may induce proinflammatory cytokines and adherent molecules by HGF, leading to the exacerbation of periodontal disease.

IL-1β induces the activation of Akt by 473 serine phosphorylation, in a PI-3K-dependent manner, and the inhibition of Akt prevents IL-1β-mediated differential embryo-chondrocyte expressed gene 1 (DEC1) and HIF-1α induction in HGF. Although IL-1β induced the ex-
pression chemokine CXCL10 and attached factor ICAM-1, in synergy with oncostatin M [36], the inhibition of CXCL10 by SE was mild (Table 2). This suggests that chemokines may not be tightly involved in the anti-inflammatory action of SE.

We have previously shown that SE and lignin–carbohydrate, both extracted by alkaline solution, showed potent anti-viral activity. Their anti-viral potential [37–43] is two-fold higher than that of tannins and flavonoids [44–46]. Recently, SE instantly inactivated both HSV and HIV, and their chemotherapeutic index is much higher than povidone-iodine. This suggests the superiority of SE over povidone-iodine for mouth wash for preventing virus infection [10]. A combination of presently described anti-inflammatory activity and potent anti-viral activity may synergistically improve the oral environment.

In conclusion, the present study demonstrated, for the first time, that the anti-inflammatory action of SE is mediated via various metabolic pathways for cell survival, apoptosis, and leukocyte recruitment. Since SE showed potent anti-inflammatory activity, in addition to its potent anti-viral activity reported previously, the applicability of SE to oral inflammation and virally induced oral diseases was suggested.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10102100/s1, Figure S1: Anti-inflammatory activity of p-coumaric acid and other lower molecular polyphenols, Figure S2: Changes in the intracellular concentrations of methionine sulfoxide, 5-oxoproline and SAM after addition of IL-1β (3 ng/mL) and TiO2 nanoparticles in HGE. Table S1: Original data for Figure 2, Table S2: Original data with SD values for Table 1, Table S3: Original data for Table 2, Table S4: Intracellular and extracellular concentration of p-coumaric acid.

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Data Availability Statement: We have provided our previous data that support our present findings in the supplementary materials Figure S2.

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