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Shosaikoto increases calprotectin expression in human oral epithelial cells

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Background and Objective: Oral epithelial cells help to prevent against bacterial infection in the oral cavity by producing antimicrobial peptides (AMPs). A broad-spectrum AMP, calprotectin (a complex of S100A8 and S100A9 proteins), is expressed by oral epithelial cells and is up-regulated by interleukin-1α (IL-1α). Shosaikoto (SST) is a traditional Japanese herbal medicine that has immunomodulatory effects and is reported to enhance the levels of IL-1α in epithelial cells. The purpose of this study was to investigate the effect of SST on the expression of calprotectin and other AMPs through the regulation of IL-1α in oral epithelial cells.

Material and Methods: Human oral epithelial cells (TR146) were cultured with SST (at concentrations ranging from 10 to 250 μg/mL) in the presence or absence of anti-IL-1α or IL-1 receptor antagonist. The expression of S100A8- and S100A9-specific mRNAs was examined by northern blotting. Calprotectin expression and IL-1α secretion were investigated by immunofluorescent staining or ELISA. The expression of other AMPs and IL-1α was analyzed by RT-PCR and by quantitative real-time PCR.

Results: Shosaikoto (25 μg/mL) significantly increased the expression of S100A8- and S100A9-specific mRNAs and calprotectin protein. Shosaikoto increased S100A7 expression, but had no effect on the expression of other AMPs. The expression of IL-1α-specific mRNA and its protein were slightly increased by SST. A neutralizing antibody against IL-1α or IL-1 receptor antagonist inhibited SST up-regulated S100A8/S100A9 mRNA expression.

Conclusion: These results suggest that SST increases the expression of calprotectin and S100A7 in oral epithelial cells. In response to SST, up-regulation of calprotectin may be partially induced via IL-1α.

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Oral epithelium functions as a physical barrier against many microbes. To prevent microbial infection, oral epithelial cells protect themselves by producing antimicrobial peptides (AMPs), including calprotectin, defensin, secretory leukocyte protease inhibitor (SLPI) and cathelicidin (1–4). These AMPs have broad-spectrum antimicrobial activity against bacteria, fungi and viruses, and play an important role in innate immunity of the oral cavity.

Calprotectin, a complex of S100A8 and S100A9 proteins, is one of the AMPs expressed in healthy oral and gingival epithelium, and has zinc-chelating antimicrobial activity (1,5,6). We previously found that calprotectin inhibited the growth of *Porphyromonas gingivalis* and the adhesion of *P. gingivalis* to epithelial cells (R. Kido and J. Kido, unpublished data, 7), suggesting that calprotectin may prevent infection with periodontopathic bacteria. Calprotectin is produced by human gingival epithelial cells and its expression is regulated by pro-inflammatory

cytokines and modulators of epithelial cell differentiation (8,9).

Shosaikoto (SST) is a Japanese and Chinese (Xiao Chai Hu Tang) traditional herbal medicine and has been prescribed for chronic liver diseases such as hepatitis, liver fibrosis and cirrhosis, and for respiratory tract diseases (10–14). Shosaikoto consists of aqueous extracts from seven herbs including Bupleurum root, Pinellia tuber, Scutellaria root, Jujube fruit, Ginseng root, Glycyrrhiza root and Ginger rhizome, and more than 15 active ingredients are contained in SST extracts (12,13,15). Shosaikoto has immunomodulatory, anti-oxidant, anti-inflammatory and anti-proliferative effects, and is reported to regulate the production of some cytokines and growth factors, including interferon-γ, interleukin (IL)-6 and granulocyte–macrophage colony-stimulating factor in liver or lung (12,16–18). Shosaikoto, or one or more ingredients, stimulates natural killer cell activity, suppresses replication of HIV and coxsackie B virus, and inhibits growth of carcinoma cells by inducing apoptosis (16,19–21).

Matsumoto *et al.* (22) reported that SST promotes the expression of IL-1α and IL-1α receptor (IL-1R1) in human epidermal keratinocytes *in vitro*. We showed that IL-1α, a cytokine constitutively expressed in epithelial cells, up-regulates the expression of calprotectin in human normal gingival keratinocytes and in human epidermal keratinocytes (8,9). We now hypothesize that SST increases the level of expression of AMPs in epithelial cells via IL-1α. In the present study, we investigated the effect of SST on the expression of calprotectin and other AMPs in human oral epithelial cells, and examined whether SST up-regulates calprotectin expression via IL-1α.

Material and methods

Cell culture

TR146 cells, an oral epithelial cell line originally isolated from a human buccal carcinoma, were seeded at a density of 0.5×10^4 cells/cm^2 and cultured in Ham's F12 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL of penicillin and 100 μg/mL of streptomycin. For RNA analyses, TR146 cells were cultured for 3.5 d, washed and then cultured for 12 h in culture medium supplemented with 2% FBS. Shosaikoto (0–250 μg/mL) was added to the culture and the cells were cultured for another 6–72 h. In neutralizing experiments, cells were pre-treated with anti-human IL-1α (1:100 dilution: IgG fraction of anti-human IL-1α; Rockland, Gilbertsville PA, USA) for 8 h, or with 100 ng/mL of IL-1 receptor antagonist [recombinant human IL-1ra/IL-1F3: (rhIL-1ra); R&D Systems, Minneapolis, MN, USA] for 1 h, and then cultured with

Table 1. Primers used in RT-PCR and quantitative real-time PCR

Gene	Primer
ADM	For: 5′-ATGAAGCTGGTTTCCGTC-3′
	Rev: 5′-TGTGGCTTAGAAGACACC-3′
AZU1	For: 5′-GACTGGATCGATGGTGTTCTC-3′
	Rev: 5′-CAGAGGAGAGATCGGCTTCTT-3′
CAMP	For: 5′-GGTCCTCAGCTACAAGGAAGC-3′
	Rev: 5′-ATCCTCTGGTGACTGCTGTGT-3′
CST3	For: 5′-AGATCGTAGCTGGGGTGAACT-3′
	Rev: 5′-GCACAGCGTAGATCTGGAAAG-3′
DEFB1	For: 5′-TGAGTGTTGCCTGCCAGTCGC-3′
	Rev: 5′-CTTGAATTTTGGTAAAGATCG-3′
DEFB4	For: 5′-CCAGCCATCAGCCATGAGGGT-3′
	Rev: 5′-GGAGCCCTTTCTGAATCCGCA-3′
DEFB103A	For: 5′-CCTTTTCATCCAGTCTCAGCG-3′
	Rev: 5′-GCGTCGAGCACTTGCCGATCT-3′
GAPDH	For: 5′-TCCACCACCCTGTTGCTGTA-3′
	Rev: 5′-ACCACAGTCCATGCCATCAC-3′
GAPDH[a]	For: 5′-GACCCCTTCATTGACCTCAACTAC-3′
	Rev: 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′
G-CSF	For: 5′-CTGTGTGCCACCTACAAG-3′
	Rev: 5′-GCCATTCCCAGTTCTTCC-3′
IL-1α	For: 5′-GTCTCTGAATCAGAAATCCTTCTATC-3′
	Rev: 5′-CATGTCAAATTTCACTGCTTCATCC-3′
IL-1α[a]	For: 5′-ATCAGTACCTCACGGCTGCT-3′
	Rev: 5′-TGGGTATCTCAGGCATCTCC-3′
IL-1R1	For: 5′-TGCCGCTCTTCTGTCATCCCGCTC-3′
	Rev: 5′-GGGGGGACCGTTATTGACCTGAAA-3′
IL-6	For: 5′-ATGAACTCCTTCTCCACAAGCGC-3′
	Rev: 5′-GAAGAGCCCTCAGGCTGGACTG-3′
LCN2	For: 5′-TGTCACCTCCGTCCTGTTTAG-3′
	Rev: 5′-TCTCCCGTAGAGGGTGATCTT-3′
MUC5B	For: 5′-TGCAATCAGCACTGTGACATTGAC-3′
	Rev: 5′-TTCTCCAGGGTCCAGGTCTCATTC-3′
S100A7	For: 5′-TGCTGACGATGATGAAGGAG-3′
	Rev: 5′-ATGTCTCCCAGCAAGGACAG-3′
S100A8	For: 5′-GCTGGAGAAAGCCTTGAACTC-3′
	Rev: 5′-CCACGCCCATCTTTATCACCA-3′
S100A9	For: 5′-TCGCAGCTGGAACGCAACATA-3′
	Rev: 5′-AGCTCAGCTGCTTGTCTGCAT-3′
SLPI	For: 5′-CAGAGTCACTCCTGCCTTCAC-3′
	Rev: 5′-CTCTGGCACTCAGGTTTCTTG-3′
TNF-α	For: 5′-ATGAGCACTGAAAGCATGATC-3′
	Rev: 5′-TCACAGGGCAATGATCCCAAAGTAGACCTGCCC-3′

[a]Primer for real-time PCR.
ADM, adrenomedullin; AZU1, azurocidin 1; CAMP, cathelicidin; CST3, cystatin C; DEFB1, β-defensin 1; DEFB4, β-defensin 2; DEFB103A, β-defensin 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1α, interleukin-1α; IL-R1, interleukin-1 receptor; IL-6, interleukin-6; LCN2, lipocalin 2; MUC5B, mucin 5; SLPI, secretory leukocyte protease inhibitor; TNF-α, tumor necrosis factor-α.
For, forward; Rev, reverse.

SST (50 μg/mL) for 24 or 48 h. For immunofluorescence analysis, cells were cultured for 24 h in culture medium and then incubated with 25 μg/mL of SST for 24 h. For ELISA analyses, TR146 cells were cultured for 3.5 d, then for 12 h in culture medium supplemented with 2% FBS and then for 48 h without (control) or with SST (25 μg/mL). The medium supplemented with SST was changed every day. The influence of SST on cell viability and cell proliferation after addition of a drug was investigated by Trypan Blue exclusion and the cell counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Shosaikoto was supplied by Tsumura & Co. (Tokyo, Japan).

RNA isolation and northern blot analysis

Total RNA was isolated from the cultured cells using the RNAiso® reagent (TaKaRa Bio Inc., Otsu, Japan). Ten micrograms of total RNA was electrophoretically separated on 6% formaldehyde–1% agarose gels and then transferred to Hybond N^+ membranes (GE Healthcare Bio-Science Co., Piscataway, NJ, USA). The cDNA probes for S100A8, S100A9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by the PCR amplification method using the primers listed in Table 1. The synthesized cDNA probes were labeled with [α-^{32}P]dCTP using the BcaBest labeling kit (TaKaRa Bio Inc.). Prehybridization was performed for 2 h at 42°C in 50% formamide, 5 × SSPE (SSPE in a buffer containing NaCl, NaH_2PO_4 $2H_2O$ and EDTA), 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate and 200 μg/mL of salmon sperm DNA. Hybridization was performed for 12 h at 42°C in the same solution containing ^{32}P-labeled cDNA probes. The membrane was washed three times, at 65°C, in 2 × SSPE containing 0.1% sodium dodecyl sulfate and was then exposed to an imaging plate (Fuji Photofilm Co., Tokyo, Japan). The hybridization signals were visualized and analyzed using a BAS 2000 bio-imaging analyzer (Fuji Photofilm) and normalized to signals of GAPDH. The signal ratio was compared between SST and control samples and expressed as relative mRNA expression.

Immunofluorescence

The cultured TR146 cells were washed in phosphate-buffered saline (PBS), fixed with periodate–lysine–paraformaldehyde solution for 4 h at 4°C, washed three times in PBS and then blocked with 4% bovine serum albumin for 40 min. After blocking, the cells were washed with PBS and reacted with rabbit anti-human calprotectin (1:500 dilution, supplied by Dr Magne K. Fagerhol, CalproAS, Oslo, Norway) for 30 min at room temperature and then with goat anti-rabbit IgG conjugated with Alexa Fluor® 546 (1:1000 dilution; Molecular Probes, Inc., Eugene, OR, USA) for 30 min at room temperature in the dark. Cells were washed in PBS and then observed using fluorescence and phase-contrast microscopes (Olympus IX-71; Olympus; Tokyo, Japan).

ELISA

TR146 cells were cultured with or without SST, anti-IL-1α or rhIL-1ra, after which the cultured medium was collected and mixed with protease inhibitors (medium fraction). The

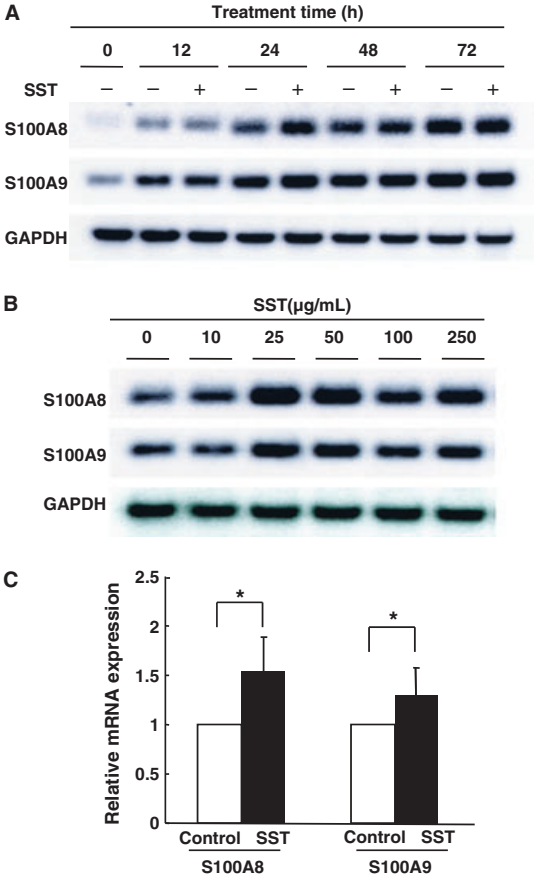

Fig. 1. Shosaikoto (SST) modulates the expression of S100A8 and S100A9 mRNAs in TR146 cells. The expression of S100A8- and S100A9-specific mRNAs was analyzed by northern blotting. TR146 cells (0.5×10^4 cells/cm^2) were cultured for 4 d and then (A) with SST (25 μg/mL) for a further 12–72 h or (B) with SST (0–250 μg/mL) for a further 24 h. (C) Quantification of S100A8- and S100A9-specific mRNA in TR146 cells following incubation with 25 μg/mL of SST for 24 h. The expression of S100A8- and S100A9-specific mRNAs was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ratio of the mRNA signal between SST and the control was expressed as 'relative mRNA expression'. The data shown are the mean ± standard deviation (SD) of a total of 13 RNA samples from triplicate or duplicate dishes in five independent experiments (*$p < 0.01$).

cultured cells were scrabbled in 10 mM Tris–HCl (pH 7.4) containing protease inhibitors and disrupted by sonication in ice water (cell fraction). The protease inhibitors used were phenylmethylsulfonyl fluoride (1 mM), leupeptin (1 μg/mL), *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (1 μg/mL), *N*-a-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (1 μg/mL) and pepstatin (1 μg/mL). The amount of calprotectin in the cell and medium fractions was determined by sandwich ELISA using the human calprotectin ELISA test kit (HyCult Biotechnology BV, Uden, the Netherlands), and IL-1α in the culture medium was measured using the Quantikine® Human IL-1α/IL-1F1 Immunoassay (R&D Systems) according to each manufacturer's instructions. The amount of calprotectin was normalized to the amount of DNA in the disrupted cell samples. The amount of DNA was determined fluorometrically according to the method of Labarca & Paigen (23).

RT-PCR and quantitative real-time PCR

Total RNA was isolated from cultured cells using the RNAiso® reagent, and cDNA was synthesized from 1 μg of the RNA sample using ReverTra Ace-α-® (Toyobo, Osaka, Japan). The cDNA was added to the PCR mixture, which contained primers (Table 1), dNTPs, TaKaRa Taq™ HS (TaKaRa Bio Inc.) and PCR buffer, and was amplified for 30–40 cycles under the following conditions: denaturation at 94°C for 1 min; annealing at 55–64°C for 1 min; and extension at 72°C for 1 min. The PCR products were analyzed by electrophoresis. The AMPs examined by RT-PCR analysis included adrenomedullin, azurocidin 1, cathelicidin, cystatin C, β-defensin 1, β-defensin 2, β-defensin 3, lipocalin 2, mucin 5, S100A7 and SLPI. The expression of IL-1α, IL-1R1, IL-6, tumor necrosis factor-α (TNF-α) and granulocyte colony-stimulating factor (G-CSF) mRNAs was also investigated by RT-PCR. For quantitative real-time PCR, 10 ng of RNA (IL-1α) or 1 ng of RNA (GAPDH) was mixed with SYBR RT-PCR buffer, prime script RT enzyme and Ex Taq HS contained in the one-step SYBR® PrimeScript® RT-PCR kit (TaKaRa Bio Inc.) and IL-1α primers. The reaction was performed at 42°C for 5 min and at 95°C for 10 s once (reverse transcription reaction), and for 40 cycles at 95°C for 5 s and at 60°C for 30 s (PCR reaction) using the Thermal Cycler Dice® Real Time System (TaKaRa Bio Inc.). The amount of IL-1α mRNA was normalized to that of GAPDH mRNA.

Statistical analysis

The statistical significance of the difference between two groups (control and SST, or SST and SST + anti-IL-1α or SST + rhIL-1ra) was determined using the unpaired Student's *t*-test. A *p*-value of < 0.05 was considered significant. Data were expressed as the mean value ± standard deviation (SD) of the number of samples.

Results

SST up-regulates the expression of calprotectin mRNA in TR146 cells

To investigate the effect of SST on the expression of S100A8- and S100A9-specific mRNAs, northern blot analysis was performed (Fig. 1). When TR146 cells were cultured with or without SST (25 μg/mL) for 0–72 h, expression of S100A8 and S100A9 mRNAs was first observed above background at 12 h and plateaued by 24 h (Fig. 1A). When the cells were cultured with 0–250 μg/mL of SST for 24 h, expression of S100A8- and S100A9-specific mRNA was maximal in response to 25–50 μg/mL of SST (Fig. 1B). At 25 μg/mL of SST, expression of S100A8 mRNA (~1.5-fold) and S100A9 mRNA (~1.3-fold) increased significantly ($p < 0.01$;

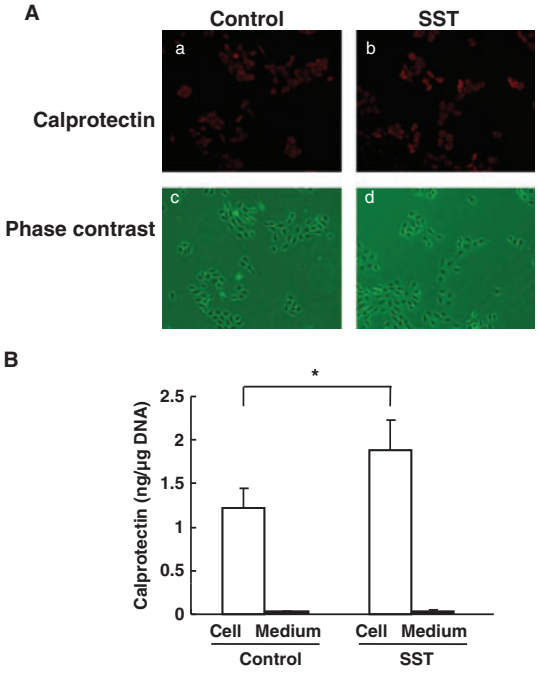

Fig. 2. Shosaikoto (SST) modulates calprotectin protein expression. (A) TR146 cells (0.5×10^4 cells/cm^2) were cultured for 1 d and then with or without SST (25 μg/mL) for one more day. Cells were immunostained with rabbit anti-human calprotectin antibody and detected using goat anti-rabbit IgG conjugated to Alexa Fluor 546 (red). Calprotectin expression in TR146 cells (a) without SST and (b) with 25 μg/mL of SST; (c) and (d) show the same fields as (a) and (b) in phase contrast (magnification ×100). (B) Cells were cultured for 4 d and then with or without SST (25 μg/mL) for 48 h. The amount of calprotectin in the cell and medium fractions was determined using ELISA. The concentration of calprotectin is expressed as nanogram per microgram cell DNA ± standard deviation (SD) from a total of five protein samples in three independent experiments (*$p < 0.05$).

n = 13 samples) compared with non-treated controls (Fig. 1C).

The viability of cells treated with 0–250 μg/mL of SST was > 94% at 24 and 48 h and > 90% at 72 h. Shosaikoto did not appear to affect the cell proliferation or growth rate for 24–72 h after the addition of 25–50 μg/mL of SST.

SST up-regulates expression of calprotectin protein in TR146 cells

TR146 cells expressed calprotectin in the cytoplasm, as detected using immunofluorescence (Fig. 2A). In cells treated with 25 μg/mL of SST, calprotectin expression appeared to be greater than that of non-treated cells (control). Nonimmune rabbit serum controls showed no detectable fluorescence (data not shown).

Shosaikoto (25 μg/mL) increased the amount of calprotectin protein in the cell fraction from 1.22 ng/μg of cell DNA to 1.80 ng/μg of cell DNA (Fig. 2B). The concentration of calprotectin in the medium fraction was approximately 2–2.5% of that in the cell fraction and there was no significant difference between the medium fractions from control (0.029 ng/μg of cell DNA) and SST-treated cell culture (0.036 ng/μg of cell DNA).

SST regulates the expression of AMPs and cytokines

In response to SST (25 μg/mL), S100A7 mRNA expression increased, but the expression of other AMPs, including adrenomedullin, cathelicidin, cystatinC, β-defensin 2, lipocalin 2 and SLPI was unaffected (Fig. 3). Azurocidin 1, β-defensin 1, β-defensin 3 and mucin 5 mRNAs were not detected in either nontreated or SST-treated TR146 cells.

Independently of the presence of SST, the expression of IL-1α mRNA increased from 6 to 12 h, whereas the expression of IL-1R1 mRNA appeared to be unchanged during 6–24 h (Fig. 4). Shosaikoto induced a slight increase of IL-1α mRNA expression at 6 h (Fig. 4A), and significantly increased the expression of IL-1α mRNA (6 h) and IL-1α protein in the culture medium (12 h) by approximately 1.5-fold and 1.2-fold relative to the control, respectively (Fig. 4B,C). In contrast, SST had no effect on the expression of IL-6, TNF-α and G-CSF mRNAs after treatment for up to 24 h.

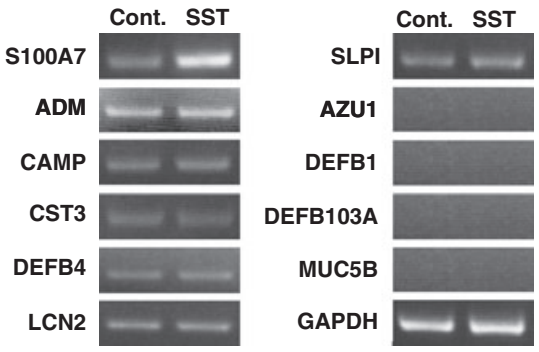

Fig. 3. Shosaikoto (SST) modulates the expression of S100A7 mRNA in TR146 cells. Subconfluent cells cultured for 4 d were incubated with or without SST (25 μg/mL) for 24 h. After RNA isolation, the expression of antimicrobial peptide (AMP) mRNAs, including adrenomedullin (ADM), azurocidin 1 (AZU1), cathelicidin (CAMP), cystatin C (CST3), β-defensin 1 (DEFB1), β-defensin 2 (DEFB4), β-defensin 3 (DEFB103A), lipocalin 2 (LCN2), mucin 5 (MUC5B), S100A7 and secretory leukocyte protease inhibitor (SLPI) mRNAs were analyzed by RT-PCR using the primers and PCR cycles shown in Table 1. These results represent one of three independent experiments. Cont., control.

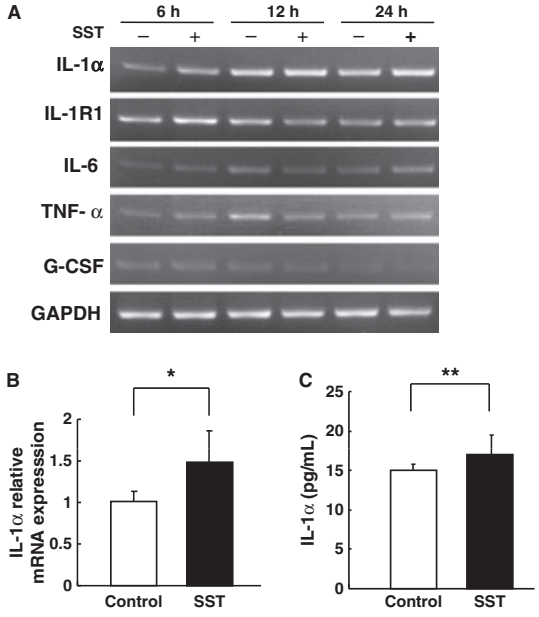

Fig. 4. Shosaikoto (SST) modulates the expression of cytokines in TR146 cells. Subconfluent cells cultured for 4 d were incubated with or without SST (25 μg/mL) for 6, 12 or 24 h. RNA isolation and PCR reactions are described in the Material and methods. The expression of cytokine mRNAs (A), including interleukin-1α (IL-1α), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and granulocyte colony-stimulating factor (G-CSF) mRNAs, and interleukin-1α receptor (IL-1R1) mRNA, was analyzed using RT-PCR. The agarose gel is representative of three independent experiments. The expression of IL-1α mRNA in the cells treated with SST for 6 h (B) was detected by quantitative real-time PCR and normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The data are mean ± standard deviation (SD) of nine independent RNA samples (*$p < 0.01$). The IL-1α concentration in the medium cultured for 12 h (C) was determined by ELISA, as described in the Material and methods. The data are the mean ± SD of nine independent protein samples (**$p < 0.05$).

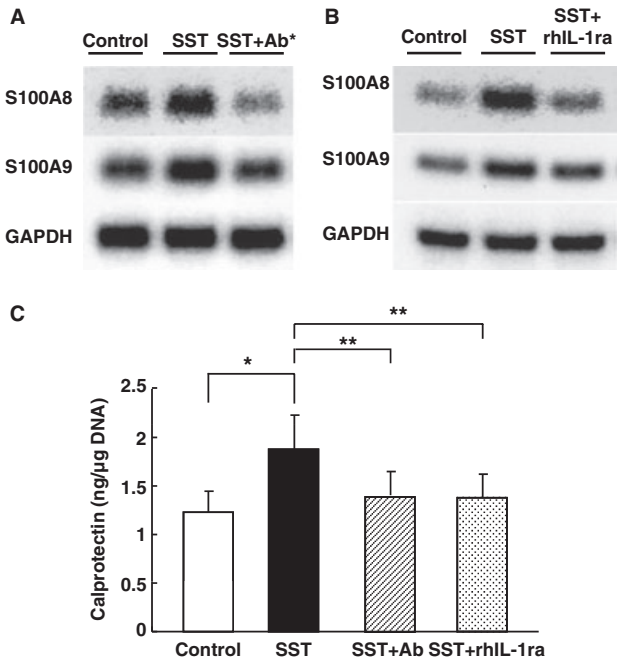

Fig. 5. Anti-interleukin-1α (IL-1α) and IL-1 receptor antagonist (IL-1ra) inhibit Shosaikoto (SST)-induced up-regulation of calprotectin (a complex of S100A8 and S100A9 proteins). Subconfluent cells were pretreated with the IgG fraction of anti-human IL-1α (Ab*: 1:100 dilution) for 8 h or with recombinant human (rh)IL-1ra (100 ng/mL) for 1 h, and cultured with SST (50 μg/mL) for 24 h (northern blot analysis) or 48 h (ELISA). After RNA isolation, the expression of S100A8 and S100A9-specific mRNAs was analyzed by northern blotting (Fig. 5A,B). These results were verified using three independent RNA samples. The amount of calprotectin protein was determined using ELISA (Fig. 5C). The data are the mean ± SD of three independent protein samples (*$p < 0.01$, **$p < 0.05$).

Anti-IL-1α and rhIL-1ra inhibit SST-induced up-regulation of calprotectin expression

To determine whether IL-1α is related to SST-induced up-regulation of calprotectin, anti-IL-1α and rhIL-1ra were tested (Fig. 5). Shosaikoto up-regulated the expression of S100A8/S100A9 mRNAs. Addition of anti-IL-1α inhibited the SST-induced up-regulation of S100A8/S100A9 mRNA expression (Fig. 5A). Incubation of cells with rhIL-1ra also suppressed SST-induced S100A8/S100A9 mRNA expression (Fig. 5B). Neither anti-IL-1α nor the IL-1ra affected expression of S100A8/S100A9 mRNA in cells cultured without SST (data not shown). Furthermore, anti-IL-1α and rhIL-1ra significantly inhibited SST-induced expression of cytosolic calprotectin protein (Fig. 5C; control, 1.22 ng/μg of cell DNA; SST, 1.88 ng/μg of cell DNA; SST + anti-IL-1α, 1.39 ng/μg of cell DNA; and SST + rhIL-1ra, 1.37 ng/μg of cell DNA).

Discussion

Shosaikoto is a traditional herbal medicine that has been used to treat liver and lung diseases, viral infection and tumors, and shows immunomodulatory, anti-inflammatory, anti-proliferative and anti-oxidant activities (10–13). The immunomodulatory function of SST induces antigen phagocytosis in macrophages and promotes antigen presentation in lymphocytes (24) and increases natural killer activity in peripheral blood (19). Shosaikoto augments immunological functions by inducing G-CSF, granulocyte–macrophage colony-stimulating factor, IL-10 and IL-12 in hepatitis patients with a low level of cytokines (17,25–28). For the first time, we now report that SST selectively increases expression of the S100 family of proteins, including S100A7, S100A8 and S100A9. S100A8 and S100A9 (calprotectin) contribute to innate mucosal keratinocyte and gingival immunity, suggesting that SST may be efficacious in augmenting innate immunity.

Consistent with the response of normal skin and oral mucosal keratinocytes (22, our result), SST (25 μg/mL) increased the expression of IL-1α mRNA in TR146 cells at 6 h and the expression of IL-1α protein in the culture medium at 12 h, followed by elevation of calprotectin mRNA expression about 18 h later. Interleukin-1α is constitutively expressed by epithelial cells and regulates the proliferation and differentiation of epithelial cells in an autocrine manner (29–31). We previously showed that IL-1α increased the expression of S100A8/S100A9 mRNAs and calprotectin protein in normal human gingival keratinocytes (8,9), and the expression of β-defensin 2, SLPI and lipocalin 2, as well as S100A7, S100A8 and S100A9, in human epidermal (skin) keratinocytes (32). Differences in the AMP response to IL-1α may be cell type-specific. Furthermore, AMP expression in response to IL-1α in epithelial cells may differ in the presence and absence of SST. Collectively, however, SST appears to up-regulate the expression of calprotectin (S100A8/S100A9) and S100A7 through an IL-1α-mediated pathway (Fig. S1).

Shosaikoto contains several compounds with pharmacological potential, including baicalein, baicalin, wogonin, wogonin-7-*O*-glucuronoside, ephedrine, ginsenoside Rb1, ginsenoside Rg1, glycyrrhizin, liquiritin, liquiritigenin, 6-gingerol, 6-shogaol, zingerone and several saikosaponins (12,13,15). Some of the compounds are reported to have immunomodulatory and anti-inflammatory activities. For example, wogonin/wogonin-7-*O*-glucuronoside and ginsenoside Rb1/ginsenoside Rg1 inhibit CD8 T-cell proliferation and modulate the T helper 1/T helper 2 cell balance (33,34). Baicalin and glycyrrhizin elevate the level of cytokines, including IL-1β, IL-6, IL-10, IL-12, TNF-α and G-CSF, in blood mononuclear cells, lymphocytes

or lung tissue (12,18). In the present study, SST had no effect on the expression of the cytokines IL-6, TNF-α and G-CSF, but did up-regulate IL-1α, S100A7, S100A8 and S100A9. Furthermore, Baicalin and Ginsenoside Rb1 increased the expression of S100A8 and S100A9 mRNAs (Fig. S2), but Baicalein and Wogonin decreased their expression in TR146 cells (data not shown). New functions for some compounds in SST are likely.

Psoriasin (S100A7) and calprotectin (S100A8/S100A9) are expressed in healthy epidermal or mucosal epithelial cells. Their levels elevate in inflammatory tissues and are increased by pro-inflammatory cytokines and stimulators of keratinocyte differentiation (1,6,8,9,32,35,36). By contrast, traditional medicines had not been reported to increase the expression of these AMPs. Shosaikoto ameliorated lipopolysaccharide-induced lung injury and experimental liver injury by plural pharmaceutical effects such as cytoprotective, immunomodulatory, anti-inflammatory and apoptosis-inducing functions (11,21,37); SST gargle significantly suppressed the incidence of stomatitis in patients treated with chemotherapy (38); and, furthermore, SST decreased lipopolysaccharide-induced prostaglandin E_2 production in human gingival fibroblasts (39). Shosaikoto may affect wound healing by regulating S100A7, S100A8 and S100A9 expression. Whether herbal medicines, such as SST, can be used for periodontal treatment is not known, but there are some promising characteristics. Conth Su and Chi Tong Ning have direct antibacterial activity, and Conth Su promotes the regeneration of epithelium in hamsters with experimental periodontitis (40). Shosaikoto also appears to show antimicrobial activity by inducing AMPs and anti-inflammatory action (our result, 41). Traditional herbal medicines generally show few side effects and do not induce bacterial resistance. Shosaikoto therefore may prove useful in the treatment of periodontal diseases and other oral infections by regulating AMP expression and other complementary activities in oral epithelial cells.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of IL-1α on S100A8/S100A9 mRNA expression in TR146 cells.

Figure S2. Effect of Baicalin and Ginsenoside Rb1 on the expression of S100A8/S100A9 mRNAs (northern blotting).

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