Sopungyangjae-Tang Inhibits Development of Dermatitis in Nc/Nga Mice

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Sopungyangjae-Tang (SYT) is a traditional Korean decoction used for the treatment of dermatitis. The aim of this study was to confirm whether or not SYT has a preventive effect on the development of atopic dermatitis in dinitrochlorobenzene-applied Nc/Nga mice. SYT was administered orally to Nc/Nga mice, which led to the remarkable suppression of the development of dermatitis, as determined by a histological examination and the serum IgE levels. Moreover, SYT inhibited the production of thymus- and activation-regulated chemokine (TARC) and its mRNA expression in a keratinocyte cell line, HaCaT, which had been stimulated with tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Activation of the nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) is one of the key steps in the signaling pathways mediating induction of TARC. In this study, SYT selectively suppressed NF-κB activation, which may be essential for TARC expression in TNF-α/IFN-γ treated keratinocytes. The inhibitory effect of SYT on NF-κB activation and TARC production might be associated with the anti-dermatitic effects of SYT.

Keywords: anti-dermatitic activity – atopic dermatitis – NF-κB – Sopungyangjae-Tang – TARC

Introduction

Atopic dermatitis is a clinical syndrome that is characterized by pruritic skin lesions that are distinguished by infiltrating lymphocytes, macrophage and granulated mast cells (1,2). Atopic dermatitis is a common disease with an increasing incidence in industrialized countries. Although topical steroid therapy is used to manage this disease (3), many patients suffer from the serious side effects of steroid ointments. Hence, a great deal of effort has been directed toward identifying safer compounds or herbal remedies that can inhibit the development of atopic dermatitis.

Some traditional Korean herbal prescriptions have been clinically applied as curative agents against incurable chronic diseases. Sopungyangjae-Tang (SYT) is widely used in Korea for the treatment of dermatitis. SYT is a decoction consisting of 15 medicinal plants, Angelicae Gigantis Radix, Moutan Cortex, Rehmanniae Radix, Glycyrrhizae Radix, Linocerae Flos, Ledebouriellae Radix, Spirodelae Herba, Forsythiae Fructus, Paeoniae Radix Rubra, Cnidii Rhizoma, Schizonepetae Herba, Bombyx Batryticatus, Menthae Herba, Bubali Cornu and Scutellariae Radix.

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IL-4 (5). TARC produced from basal keratinocytes play a significant role in recruiting Th2-type lymphocytes to dermatitic skin lesions and the subsequent deterioration of dermatitis (6,7).

It has been reported that Nc/Nga mice develop dermatitis with depilation and hemorrhage when housed in conventional conditions but not in specific pathogen-free conditions (8). This study investigated the protective effect of SYT on development of dermatitis in Nc/Nga mice that had been topically applied with 1-chloro-2,4-dinitrobenzene (DNCB). The effect of SYT on TARC production in a keratinocyte cell line, HaCaT, was also examined to elucidate a possible mechanistic basis for the anti-dermatitic effects of SYT.

Methods

Mice

The Institutional Animal Care and Utilization Committee of Chosun University approved all animal procedures. Male Nc/Nga and ICR mice (weighing 25 g) were purchased from Joong-Ang Experimental Animals Co. (Seoul, Korea), placed in cages at a temperature between 20 and 23°C with a 12 h light and dark cycle and a relative humidity of 50%. Animals were given commercial mouse chow (Purina, Korea) and water ad libitum. Controlled dermatitis was induced by topically applying 0.1% DNCB dissolved in acetone/olive oil (1:3), to the hair-removed back of mice 3 times per week (Monday, Wednesday and Friday) for 10 weeks. Mice were then housed for 3 days without any further treatment. In the case of the SYT-treated group, SYT was orally administered at a dose of 20, 40 or 60 mg/mouse (6 times per week for 12 weeks) in their drinking water. Control animals received the vehicle. Mice were sacrificed on the day of the experiment (on 73 days after first applying the DNCB) and blood was collected from the vena cava.

Preparation of Sopungyangjae-Tang

All medicinal plants were purchased from a herbal market (Daewon, Daegu, Korea) and were authenticated by Professor Sang Chan Kim, Daegu Haany University. Herbarium voucher specimens of SYT were prepared and deposited in the herbarium of the Graduate School of Oriental Medicine, Daegu Hanny University. The SYT used in this study was composed of 12 g Angelicae Gigantis Radix, 12 g Moutan Cortex, 12 g Rehmannia Radix, 4 g Glycyrrhize Radix, 4 g Linocerae Flos, 4 g Ledebourielae Radix, 4 g Spirodela Herba, 4 g Forsythiae Fructus, 4 g Paeoniae Radix Rubra, 4 g Cnidii Rhizoma, 4 g Schizonepetae Herba, 4 g Bombyx Batryticatus, 2 g Menthae Herba, 2 g Bubali Cornu and 2 g Scutellariae Radix. A water extract of SYT was prepared by boiling 1560 g SYT in 4 l of distilled water at 100°C for 3 h. The extract was centrifuged 3000 g for 3 min and the resulting supernatant was lyophilized to produce a powder (232.6 g), which was stored at 4°C.

Other Materials

Anti-phospho inhibitor-κBα(I-κBα) antibody was supplied from Cell Signaling Technology (Beverly, MA, USA). The recombinant tumor necrosis factor-α (TNF-α) and tumor necrosis factor interferon-γ (IFN-γ) were obtained from Peprotech Inc. (Rocky Hill, NJ, USA). The human TARC and mouse IgE enzyme linked-immunosorbent assay kits were purchased from R&D Systems (Minneapolis, MN, USA) and Shibayagi Co. (Gunma, Japan), respectively. 1-chloro-2,4-dinitrobenzene (DNCB) was obtained from Aldrich (Milwaukee, WI, USA). Most of the reagents used for molecular studies were purchased from Sigma (St. Louis, MO, USA).

Cell Culture

HaCaT cells, a human keratinocyte cell line, were cultured in Dulbecco’s modified Eagle’s media (DMEM) supplemented with 10% fetal calf serum (FCS), 100 μg ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin, in 5% CO₂ at 37°C. Production of TARC was stimulated by incubating HaCaT cells with 20 ng ml⁻¹ TNF-α and 20 ng ml⁻¹ IFN-γ in. The SYT extract was dissolved in sterile phosphate-buffered saline and added to the incubation medium 10 min before adding TNF-α/IFN-γ.

Enzyme Linked Immunosorbent Assay (ELISA)

Both the serum levels of IgE in the Nc/Nga mice and the supernatant levels of TARC in HaCaT cells were measured according to manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm, and the concentration was calculated from a standard curve using serially diluted IgE or recombinant TARC.

Histopathologic Examination

Skin tissues were isolated from each mouse and fixed in 10% formalin in 50 mM of a phosphate buffer (pH 7.0) for 24 h at 4°C. Skin tissues were subsequently embedded in paraffin, sectioned (4 μm), stained with hematoxylin and eosin, and examined by optical microscopy (Olympus, Tokyo, Japan). A certified pathologist analyzed and scored the samples in a blinded manner. A minimum of 2 sections per experimental animal were examined for the presence and degree of incrustation, thickening of the epidermis, epidermal necrosis, bleeding,
hyperkeratosis and inflammation of the epidermis and dermis.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using the guanidium-isothiocyanate procedure, as described elsewhere (9). Total RNA (1.0 μg) obtained from cells was reverse-transcribed using an oligo(dT) 20 mer as a primer and Moloney-murine leukemia virus reverse transcriptase (Bioneer, Eumsung, Korea) to produce the cDNAs. PCR was performed using the selective primers for the human TARC (sense primer: 5'-ACTGCTCCAGGGATGCCCATTGTTTTT-3', antisense primer: 5'-ACAAGGGATGGGATCTCCCT CACTG-3') (270 bp) and the S16 ribosomal protein (S16r) genes (sense: 5'-TCCAAGGGTCCGCTGCAG TC-3', antisense: 5'-CGTCACTTTGATGAGCCCATT C-3') (152 bp). PCR was carried out for 35 cycles using the following conditions: denaturation at 98°C for 10 sec, annealing at 60°C for 0.5 min and elongation at 72°C for 1 min. The band intensities of the amplified DNAs were compared after visualization on a UV transilluminator.

Transient Transfection and nuclear Factor-κB (NF-κB) and activator protein-1 (AP-1) Reporter Gene Assay

Cells were plated on a 12-well dish and transfected the following day. Promoter activity was determined using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Briefly, cells were transiently transfected with 1 μg of pNF-κB-Luciferase or pAP-1-Luciferase plasmid and 20 ng of the pRL-SV plasmid (Renilla luciferase expression for normalization) (Promega, Madison, WI, USA) using the Genejuice reagent (Novagen, Madison, WI, USA), and then exposed to TNF-α (20 ng ml⁻¹)/IFN-γ (20 ng ml⁻¹) for 18 h. The firefly and Renilla luciferase activities in cell lysates were measured using a luminometer (Turner Designs; TD-20, CA, USA). Relative luciferase activity was calculated by normalizing the NF-κB or AP-1 promoter-driven firefly luciferase activity to that of Renilla luciferase.

Statistics

Differences between treatment groups were examined using a paired Student’s t-test. The criterion for statistical significance was set at either P < 0.05 or P < 0.01.

Results

SYT inhibits Dermatitis Progression in Nc/Nga Mice Topically Applied with DNCB

Skin of the ICR or Nc/Nga mice bred under conventional conditions for 10 weeks was first investigated. Skin of ICR mice housed in conventional conditions showed no physical signs of dermatitis (Fig. 1A) or histological evidence of inflammatory cell infiltration (Fig. 1A). Whereas, skin of Nc/Nga mice bred in conventional conditions showed mild thickening and incrustation on the back (Fig. 1B) as well as histologically mild thickening of the epidermis and a mild infiltration of inflammatory cells in the upper dermis (Fig. 1B). In order to induce stronger and more controlled dermatitis, 250 μl of 0.1% DNCB was topically applied to the back of the mice for 10 weeks (3 times a week). The DNCB applied Nc/Nga mice showed progressive diffuse erythematous changes, scaling, lichenified areas, oozing and crusting on the back (Fig. 1C) as well as histological findings of hyperkeratosis, parakeratosis, acanthosis with varying degrees of spongiosis, exocytosis of mononuclear cells in the epidermis and infiltration of inflammatory cells into the upper dermis (Fig. 1C). These results confirm that the chronic application of DNCB can accelerate development dermatitis of Nc/Nga mice.

The protective effects of SYT against DNCB-induced dermatitis in Nc/Nga mice were examined. SYT (20, 40 and 60 mg per mice) decreased the intensity of dermatitis in the DNCB-applied Nc/Nga mice. The extent of incrustation was particularly reduced by the administration of SYT [Fig. 1D (SYT 20 mg per mice) and 1E (SYT 40 mg per mice)]. Moreover, histological studies revealed that thickening of the epidermis and infiltration of inflammatory cells were significantly lower in the SYT-treated groups [Fig. 1D (SYT 20 mg per mice) and 1E (SYT 40 mg per mice)]. Multiple analyses of the histology samples confirmed that SYT significantly reduced the indices of dermatitis compared with that by DNCB alone i.e. epidermal necrosis score: 2.8 (DNCB alone) versus 0.4 (SYT 20 mg per mouse) or 0 (SYT 40 mg per mouse) (Table 1).

SYT Inhibits Serum IgE Increase in Nc/Nga Mice Topically Applied with DNCB

IgE serum levels increased gradually with increasing age of Nc/Nga mice, which is one of the indices for the development of dermatitis (8,10,11). The application of DNCB significantly enhanced the serum IgE levels compared with the vehicle-treated group (Fig. 2). The increased serum IgE levels were lower in the SYT-treated groups. In the mice treated with 20 and 40 mg SYT, the serum level of IgE was 34 and 18% of that in the DNCB-applied mice, respectively (Fig. 2). However, the serum level of IgE in the 60 mg per mouse SYT-treated group was ~50% of that in the DNCB-applied mice, but it was not significantly different, which might be due to data variances. Overall, these results suggest that the oral administration of SYT prevents the development of dermatitis in Nc/Nga mice.
SYT Inhibits TNF-α/IFN-γ-Inducible TARC Production in HaCaT Cells

TARC is a type of secreted chemokine that attracts Th2 lymphocytes expressing CCR4 and is believed to be involved in the development of Th2-mediated inflammation such as atopic dermatitis (4,12). Previous studies have shown that TARC as well as the macrophage-derived chemokine (MDC) actively participate in the pathogenesis of atopic dermatitis in Nc/Nga mice (6). It was reported that TNF-α and IFN-γ synergistically induce TARC production in primary human keratinocytes and HaCaT cells, which might play a role in the development of atopic dermatitis (6). Because SYT efficiently suppressed the development of dermatitis in the DNCB-applied Nc/Nga mice, it was hypothesized that SYT could affect the production of TARC in HaCaT cells by TNF-α/IFN-γ.

An ELISA assay using a human TARC specific antibody showed that the level of TARC production was increased 3.2 fold by incubating cells with TNF-α (20 ng ml⁻¹)/IFN-γ (20 ng ml⁻¹) for 24h. however, this effect was not observed with TNF-α or IFN-γ alone (Fig. 3A, insert). Induction of TARC production was inhibited significantly by a SYT pretreatment (300 or 1000 μg ml⁻¹) in a concentration-dependent manner (Fig. 3A). Expression levels of TARC mRNA in HaCaT cells exposed to TNF-α (20 ng ml⁻¹)/IFN-γ (20 ng ml⁻¹) for 3h were measured in order to determine if SYT inhibits the transcription of the TARC gene. Expression of TARC mRNA was increased markedly

Table 1. Effect of SYT on pathological parameters of dermatitis in Nc/Nga mice

| Group | Parameter | IncruR | E/Thick | E/Nec | Inf/Epi |
|-------|-----------|--------|---------|-------|---------|
| ICR (Vehicle) (n = 3) | | 0.02 ± 0.03 | 0.10 ± 0.003 | 0 | 0 |
| Nc/Nga (Vehicle) (n = 3) | | 0.03 ± 0.05 | 0.10 ± 0.02 | 0 | 0 |
| Nc/Nga (DNCB) (n = 6) | | 0.31 ± 0.19 | 0.17 ± 0.03 | 2.5 ± 1.2 | 2.8 ± 0.4 |
| Nc/Nga (DNCB) + SYT 20 mg per mouse (n = 6) | | 0.02 ± 0.03 | 0.10 ± 0.01 | 1.3 ± 1.5 | 0.8 ± 1.2 |
| Nc/Nga (DNCB) + SYT 40 mg per mouse (n = 6) | | 0 | 0.10 ± 0.01 | 0.5 ± 1.2 | 0 |
| Nc/Nga (DNCB) + SYT 60 mg per mouse (n = 3) | | 0.02 ± 0.03 | 0.10 ± 0.002 | 0 | 0 |

Incrustation ratio = Total length of incrusted areas/Total length of each sample; E/Thick, epidermal thickness; E/Nec, epidermal necrosis (no necrosis, 0; mild, 1; moderate, 2; severe, 3); Inf/Epi, inflammatory cells infiltration in the epidermis (no infiltration, 0; mild, 1; moderate, 2; severe, 3); Data represent the means ± SEM of 3–6 different samples.
by TNF-α/IFN-γ, and this increase was completely reversed by the presence of 300 or 1000 μg ml⁻¹ SYT. In contrast, whereas the expression of the S16rRNA protein mRNA was unchanged (Fig. 3B). Hence, SYT might be associated with the TARC transcription pathway.

SYT Selectively Inhibits NF-κB Activation in HaCaT Cells Stimulated with TNF-α/IFN-γ

NF-κB and AP-1 are activated in cells stimulated with TNF-α and IFN-γ, which is involved in the transcriptional activation of responsive genes (13–15). The promoter region of the TARC gene contains NF-κB and AP-1 binding sites (16), and recent studies revealed that the transcriptional regulation is also related to the NF-κB or AP-1 activity (16–18). Therefore, reporter gene analyses using luciferase reporter plasmids containing the NF-κB or AP-1 binding sequences were carried out in order to determine if the suppressive effect of SYT on the induction of TARC gene occurs alongside the inhibition of NF-κB or AP-1. TNF-α/IFN-γ (20 ng ml⁻¹ each, 18 h) caused a 3.2-fold increase in NF-κB reporter activity (Fig. 4A), which was inhibited by pretreating the cells with 300 or 1000 μg ml⁻¹ of SYT for 10 min (Fig. 4A). Activation (nuclear translocation) of NF-κB is preceded by the phosphorylation and subsequent degradation of the IκBα subunit (19), and thus, we further examined phosphorylated IκBα levels in HaCaT cells. Immunoblot analysis using specific antibody showed that the phosphorylation of IκBα by TNF-α/IFN-γ (20 ng ml⁻¹ each, 10 min) were also prevented by pretreating with 1000 μg ml⁻¹ of SYT for 10 min (Fig. 4A).

However, TNF-α/IFN-γ did not increase AP-1 reporter activity in HaCaT cells. Moreover, basal AP-1 reporter activity was not inhibited by SYT. Instead, there was an increase in response to SYT 1000 μg ml⁻¹ (Fig. 4B). We also confirmed TNF-α/IFN-γ-inducible AP-1 binding activity by gel shift analysis. Holvoet et al. (20) showed that TNF-α slightly increased AP-1 DNA binding activity in HaCaT cells. In their study, basal AP-1 DNA binding in quiescent cells was detected, as described in the ‘Result’ section. In our experimental condition (serum deprivation for 12 h), the basal AP-1 DNA binding activity was also strongly found and addition of TNF-α/IFN-γ did not cause further increase in the AP-1 binding activity. Moreover, SYT did not affect AP-1 DNA binding activity, which was consistent with results of AP-1 reporter gene assays (Fig. 4B). It would be possible that inflammation signal(s)-inducible AP-1 binding was not found since the basal AP-1 activity was strong in HaCaT cells.

These results suggest that SYT selectively inhibits NF-κB activation process, and is associated with its suppressive effect on TARC induction caused by inflammatory insults.

Discussion
These results highlighted protective effects of SYT on development of dermatitis in DNCB-applied Nc/Nga mice.
The topical application of DNCB on the backs of Nc/Nga mice was an efficient tool for inducing severe and controlled dermatitis within 10 weeks. In contrast, DNCB itself did not cause any pathological changes in ICR mice (data not shown), which suggests that the dermatitis observed in our system mainly results from the DNCB-inducible potentiation of spontaneous dermatitis in Nc/Nga mice. Although multiple application of 2,4,6-trinitro-1-chlorobenzene (TNCB) to mice is a representative animal model of contact dermatitis (21), DNCB caused no dermatitis symptoms in our experimental conditions.

Some plant materials contain several chemicals with anti-allergic and anti-inflammatory activity (22). There might be anti-allergic or anti-inflammatory materials such as flavonoids, lignans and catechins in the plant sources of SYT. However, SYT contain many compounds from eleven medicinal plant sources, and the structures of components might change into new or modified compounds during the boiling step. Therefore, it is impossible to isolate all the active constituents from SYT. Nc/Nga mice topically applied with DNCB were used to determine the anti-dermatitic effect of SYT in vivo. The oral administration of SYT effectively prevented development of dermatitis, as evidenced by histology examination. Moreover, ELISA assay results confirmed that a SYT treatment inhibited increase in serum IgE level. SYT is often prescribed to patients with...

Figure 3. Effects of SYT on the TNF-α/IFN-γ-induced production of TARC. The HaCaT cells were incubated in a medium containing SYT for 10 min and treated with TNF-α/IFN-γ (20 ng ml\(^{-1}\), each) for 24 h (protein) or 3 h (mRNA). (A) Effect of SYT on the TNF-α/IFN-γ-induced production of TARC in HaCaT cells. The concentration of TARC in the medium was determined using TARC-specific ELISA assays. The upper insert in the figure shows that TARC production is maximally enhanced in the TNF-α/IFN-γ-co-treated group. The results shown represent the mean ± SD of four different samples (significant compared with control, *P<0.05, **P<0.01; significant compared with TNF-α/IFN-γ-treated group, #P<0.05, ##P<0.01). (B) The TARC mRNA expression levels were determined by RT-PCR analysis. The mRNA expression of the S16 r was comparable among the samples. These results were confirmed by repeated experiments.
atopic dermatitis in Korean oriental medicine hospitals, and these results provide the necessary scientific evidence of its clinical efficacy.

TARC functions as a selective chemoattractant and assists in recruitment and migration of Th2 cells, which express CCR4 (12). TARC antibody therapy reduces the development of allergic airway inflammation and hyper-responsiveness (23). Therefore, TARC may be an important mediator that exaggerates atopic dermatitis. The clinical and pharmacological efficacy of SYT on the inhibition of dermatitis might be associated with its inhibitory actions on TARC production in keratinocytes.
because both the release of TARC and its mRNA expression are potently blocked in HaCaT cells exposed to SYT. The promoter of the human TARC gene contains several homologous consensus sequences for the binding of transcription factors including STAT, NF-κB and AP-1 (15). Among these transcription factors, either NF-κB or AP-1 is important for transcription of the human TARC gene (16,18). In this study, SYT selectively suppressed the increased reporter activity of the NF-κB promoter in the lysates obtained from HaCaT cells treated with TNF-α/IFN-γ. Therefore, the inhibition of NF-κB by SYT may block induction of TARC in TNF-α/IFN-γ-treated keratinocytes. Because herbal medicine extracts are extremely metabolized in liver and the pharmacological activity of metabolites is distinct from that of parent herbal medicine, treatment of HaCaT cells with SYT may not reflect in vivo effects of SYT. However, considering relatively high doses of SYT for treatment of dermatitis in animal models or in clinical situations, hepatic metabolism of SYT could be saturated and unchanged constituents of SYT may affect NF-κB activities in keratinocytes.

In conclusion, chronic administration of SYT, at doses of 20–60 mg per mice per day ameliorates development of dermatitis in NC/Nga mice. These beneficial effects may at least in part be associated with the inhibitory effect of SYT on TARC production from keratinocyte in dermatitic lesions. The NF-κB inhibitory action might be a possible mechanism for anti-TARC production and anti-dermatitis effects of SYT.

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