



Effect of *Sophora flavescens* Aiton extract on degranulation of mast cells and contact dermatitis induced by dinitrofluorobenzene in mice

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ABSTRACT

Ethnopharmacological relevance: The dried root of *Sophora flavescens* Aiton (Sopora radix, SR) has long been used in traditional medicine for the treatment of fever and swelling in eastern countries.

Materials and methods: The present study investigated the anti-allergic and anti-inflammatory effects of SR using 1-fluoro-2,4-dinitrofluorobenzene (DNFB)-induced contact dermatitis mouse model and in vitro using RBL-2H3 cells.

Results: In mice, the topical application of 10 mg/mL of SR effectively inhibited enlargement of ear thickness and weight induced by repeated painting with DNFB. Topical application of SR also inhibited hyperplasia, edema, spongiosis and infiltration of mononuclear cells in ear tissue. In addition, production levels of interferon-gamma and tumor necrosis factor-alpha were decreased by SR in vivo. Finally, the release of histamine and β -hexosaminidase, and migration were inhibited by treatment with SR.

Conclusions: These data indicate the potential of SR in treating patients with allergic skin diseases and also suggest that related mechanisms are involved in anti-inflammatory action on the Th 1 skewing reaction and inhibition against recruitment and degranulation of mast cells.

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Introduction

In the theory of traditional medicine, the root of *Sophora flavescens* Aiton can clear away heat and dry dampness and purge sthenic-fire from the liver and gallbladder (Zuo et al., 2003). The dried root of *Sophora flavescens* Aiton (Sopora radix, SR) has long been used for the treatment of fever and swelling in eastern countries. Especially, SR has been used to treat patients with skin diseases such as xeroderma and pruritus (Zuo et al., 2003). Prenylated, lavandulylated or lavandulyl flavanones, quinolizidine alkaloids and pterocarpanes have been isolated from SR (Kang et al., 2000; Yamamoto et al., 2001; Liu et al., 2010a; Wu et al., 2006; Ryu et al., 2008). SR or its isolated components have various effects including anti-cancer (Liu et al., 2010b), anti-oxidation (Piao et al., 2006), anti-bacterial (Kuroyanagi et al., 1999), anti-inflammatory (Jin et al., 2010), and anti-allergic activities (Quan et al., 2008), and

could be used for mast cell-related allergic diseases (Hong et al., 2009).

Allergic contact dermatitis (CD), one of the most common occupational diseases in industrialized countries, is caused by delayed-type hypersensitivity (DTH) responses to environmental allergens (Grabbe and Schwarz, 1998). A widely-used animal model of human CD, also known as contact hypersensitivity (CHS), is the DTH response to haptens in mice (Grabbe and Schwarz, 1998; Dudeck et al., 2011). Haptens induce CHS immediately and evoke a local inflammation within hours after challenge (Dudeck et al., 2011; Enk and Katz, 1992). Mast cells (MCs) are effectors of IgE-mediated allergic responses. MCs are commonly found at sites of CHS and play central roles in immediate-type allergic reactions and inflammation (Askenase et al., 1983). CHS is dramatically reduced in the absence of MCs in mouse models of mast cell deficiency (Dudeck et al., 2011). Degranulation of MCs causes the secretion of bioactive substances such as histamine, eicosanoids, proteolytic enzymes, cytokines and chemokines. Cytokines, especially tumor necrosis factor-alpha (TNF- α), released following degranulation induce late-phase allergic reactions and inflammation by recruiting immune cells (Broide, 2001).

Based on this background, we evaluated the anti-allergic and anti-inflammatory effects of SR in vivo using a mouse model of CD and in vitro using RBL-2H3 mast cell-like leukemia cells.

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The effects of SR on ear thicknesses, weights, histopathological changes of ear tissues and cytokine levels of ear tissues were assessed in vivo. The effects on migration of MCs and release of histamine and β -hexoaminidase by activated MCs were investigated in vitro.

Materials and methods

Chemicals and reagents

1-Fluoro-2,4-dinitrofluorobenzene (DNFB), dimethyl sulfoxide (DMSO), dexamethasone (DEX), phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM/high glucose), and penicillin and streptomycin mixture solution were purchased from Hyclone (Logan, UT, USA). Cytometric bead array kit was purchased from BD Bioscience (Franklin Lakes, NJ, USA). A histamine assay kit was purchased from Oxford Biomedical Research (Rochester Hills, MI, USA).

Preparation of SR

SR was purchased from Kwangmyungdang Medicinal Herbs (Ulsan, Korea). Fifty grams of SR was immersed in 1000 mL of methanol and sonicated for 30 min, and then extracted for 24 h. The extract was filtered with Whatman filter paper no. 20 and evaporated under reduced pressure using vacuum evaporator (Eyela, Japan). The condensed extract was then lyophilized using a freeze dryer (Labconco, Kansas City, MO, USA). Finally, 6.43 g of lyophilized powder was obtained (yield, 12.9%). The methanol extract of SR (Voucher no. MH2010-004) has been deposited at the Division of Pharmacology, School of Korean Medicine, Pusan National University.

Animals

Male 6-week-old Balb/c mice were purchased from Samtaco (Incheon, Korea). Mice were housed under specific pathogen-free conditions with a 12 h light/dark cycle and free access to standard rodent food and water. All animal experiments were approved by our Animal Care and Use Committee and performed according to institutional guidelines (PNU-2010-00065).

Induction of CD and experimental design

Mice were sensitized by painting 50 μ L of DNFB (0.1%, v/v) in acetone:olive oil (AOO, 4:1) on the shaved dorsum of each animal for 3 consecutive days. Four days after sensitization, each mouse was challenged by painting 30 μ L of DNFB (0.2%, v/v) in AOO on the dorsum of both ears every 2 day. For the topical application of drugs, SR and DEX were dissolved in ethanol, filtered using a 0.45 μ m pore size syringe filter and finally diluted in AOO (ethanol:AOO, 1:4). SR solution at a final concentration of 1 or 10 mg/mL was applied onto the dorsum of both ears every 2 days. All animals except naïve mice were sensitized and challenged with DNFB. The Naïve animals were treated with vehicles (for sensitization and challenge, AOO) and painted with vehicle (for treatment, ethanol and AOO) ($n=6$). Control animals (CTL) were sensitized and challenged with DNFB in AOO, then painted with vehicle (ethanol and AOO) ($n=8$). SR treated animals were sensitized and challenged with DNFB, then painted with 1 mg/mL of SR (SR1) or 10 mg/mL of SR (SR10) ($n=8$). DEX treated animals were sensitized and challenged with DNFB, then painted with 2.5 mg/mL of DEX in ethanol and AOO. DEX was used as positive control. The experimental design is summarized in Fig. 1.

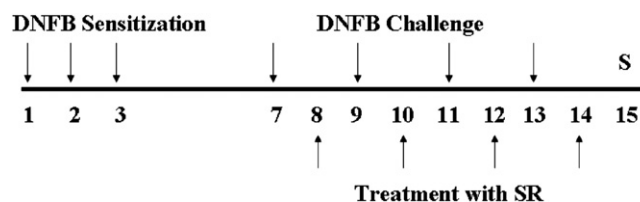


Fig. 1. Schematic of the experimental design. The experimental groups, except the Naïve group, were sensitized by painting DNFB on days 1, 2 and 3. Mice were challenged by DNFB on days 7, 9, 11 and 13. The Naïve group was treated with vehicle (AOO) in the same way. SR and DEX groups were topically applied with SR (1 mg/mL, 10 mg/mL in ethanol and AOO) or DEX (2.5 mg/mL in ethanol and AOO) on days 8, 10, 12 and 14. All animals were sacrificed on day 15.

Measurement of ear thicknesses and weights

Mice were anesthetized with 30 mg/kg of zoletil (Virbac, Carros, France) and the thickness of each right ear was measured using vernier calipers (Mitutoyo, Carros, Japan) at the end of experiment. Weights of left ear pieces (5 mm in diameter) were also measured.

Histopathological examination

After measurement of ear thicknesses and weights, ear tissues were resected and paraffin-embedded. Sections were stained with hematoxylin and eosin (H&E) for histopathological observations such as immune cell infiltration and spongiosis. Stained tissues were observed using a light microscope.

Measurement of cytokine production

At the end of experiment, resected ear tissues were lysed and homogenized with protein extraction solution (Intron bio, Daejeon, Korea) using a bullet blender (Next Advance, NY, USA) to obtain tissue lysates. Fifty micrograms of each lysate was used to measure levels of interferon-gamma (IFN- γ) and TNF- α . Cytokine levels were measured using cytometric bead array kit (BD Bioscience). All experimental procedure was conducted according to manufacturer's guidelines.

Cell culture

RBL-2H3 cells were purchased from Korean Cell Line Bank (Seoul, Korea) and grown in DMEM/high glucose supplemented with 10% FBS, 100 U/mL of penicillin and 100 μ g/mL of streptomycin at 37 °C in a humidified incubator under 5% CO₂.

Measurement of cell proliferation

Effects of SR on proliferation rates were tested using modified 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) methods. Briefly, RBL-2H3 cells were plated at a density of 1×10^5 cells/well in a 24-well plate, and SR was added to each well at a concentration of 0–400 μ g/mL in complete DMEM. After 2-h incubation, culture medium was removed and replaced, then 0.4 mL of MTT (5 mg/mL) was added to each well and the plates were incubated for 3 more hours. The remaining formazan crystals were solubilized with 0.2 mL of DMSO. The optical densities were measured using 540 nm transmission light.

Cell migration assay

The transwell migration experiments were performed in a COSTAR 24-well plate using modification of a previously-described method (Kim et al., 2008). Briefly, 200 μ L of the prepared cell

suspension (1×10^5 cells) was placed on the top of each well with the SR-containing cell medium at the bottom of the well. The assembled chamber was incubated at 37°C for 24 h in a 5% CO_2 incubator. Then, the cells on the top well were washed with PBS. Each well was added with dissociation MTT buffer and incubated at 37°C for 3 h, and then, 50 μL of DMSO was added for 5 min at room temperature. After that, 150 μL of mixture was transferred into a new 96-well plate. The Optical densities were measured using 540 nm transmission light.

β -hexosaminidase release assay

Inhibitory effects of SR on release of β -hexosaminidase from RBL-2H3 cells were measured using modification of a previously-described method (Matsuda et al., 2004). Briefly, RBL-2H3 cells were plated at a density of 2×10^4 cells/well in a 96-well plate. Cells were incubated overnight for attachment, then were treated with the indicated concentrations of SR for 1 h prior to stimulation with 50 nM of PMA plus 1 μM of A23187 at 37°C for 60 min. After stimulation, 50 μL of each sample was incubated with 50 μL of 1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide dissolved in 0.1 M citrate buffer (pH 5) in 96-well plate at 37°C for 1 h. The reaction was terminated with 200 μL /well of 0.1 M carbonate buffer (pH 10.5). The absorbance at 405 nm was measured using a Microplate reader (TECAN, Männedorf, Switzerland). The inhibition percentage of β -hexosaminidase release was calculated as the absorbance of supernatant $\div$ (absorbance of supernatant + absorbance of pellet) $\times 100$.

Histamine release assay

RBL-2H3 cells were plated at a density of 2×10^4 cells/well in a 96-well plate. Cells were incubated overnight in a complete medium then were treated for 1 h with the indicated concentrations of SR prior to stimulation with 50 nM of PMA plus 1 μM of A23187 at 37°C for 30 min. The histamine contents were measured

using a Histamine detection kit (Oxford Biochemical Research) according to the manufacturer's instructions.

Statistical analysis

All statistical comparisons were made with Student's *t*-test. The SigmaPlot version 11.0 (SYSTAT Software, San Diego, CA, USA) was used for statistical analysis. All data are presented as mean $\pm$ SD. Differences with a value of $P < 0.05$ were considered as significant.

Results

Effects of SR on ear swelling induced in CD mice

The effects of SR on ear swelling were evaluated by measuring ear thicknesses and weights. Repeated painting of DNFB induced ear swelling, which is a major feature of CD. DNFB painting increased ear thickness up to 0.59 ± 0.1 mm and ear weight was also increased up to 25.8 ± 3.5 mg in the CTL group. These increases in thickness and weight of ear tissue were inhibited effectively by treatment with SR. In mice treated with 10 mg/mL SR (SR10 group), ear thickness and weight were decreased significantly compared to those of the vehicle-treated CTL group. Ear thicknesses in the SR1 group were decreased significantly, but ear weights were only marginally decreased. However, there were no statistical significances (Fig. 2).

Effects of SR on histopathological changes of ear tissues in CD mice

There were no abnormal changes in ear tissues from the Naïve group (Fig. 3A). The epidermis in the CTL mice displayed hyperplasia, and significant edema and spongiosis were observed. In addition, marked infiltration of mononuclear cells was evident in the dermis (Fig. 3B). SR treatment diminished hyperplasia, edema

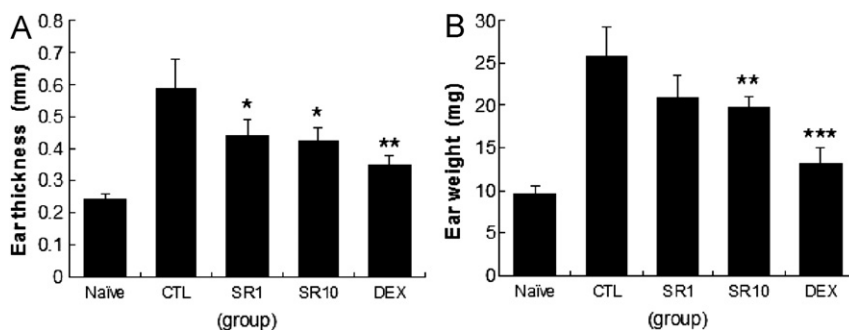


Fig. 2. Effects of SR on ear swelling in CD mice. Inhibition of ear swelling by topical application of SR was analyzed by measuring ear thicknesses using vernier calipers and weights using microbalance on day 15. Naïve, non-treated normal mice; CTL, non-treated CD mice; SR1, 1 mg/mL of SR painted CD mice; SR10, 10 mg/mL of SR painted CD mice; DEX, 2.5 mg/mL of dexamethasone painted CD mice. (A), ear thicknesses; (B), ear weights. All values represent mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. non-treated CD mice (CTL).

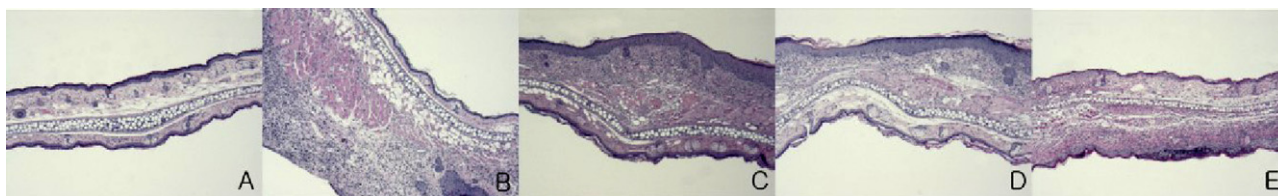


Fig. 3. Effects of SR on histopathological changes in CD mice. Ear tissues were stained with H&E and observed using a light microscope. There were no abnormal changes in ear tissues from the Naïve group (A). The tissues of CTL mice displayed hyperplasia as well as significant edema and spongiosis, and marked infiltration of mononuclear cells was observed (B). 10 mg/mL of SR treatment diminished hyperplasia, edema and spongiosis (C). 1 mg/mL of SR treatment was less effective than 10 mg/mL of SR treatment (D). There was some infiltration of immune cells in DEX group (E). All observations were made at a magnification of $\times 50$.

and spongiosis. Treatment with 10 mg/mL of SR was more effective than treatment with 1 mg/mL (Fig. 3C and D). The DEX group displayed almost normal features, and there was some infiltration of immune cells (Fig. 3E).

Effects of SR on levels of IFN- γ and TNF- α in CD mice

Repeated application of DNFB resulted in increased IFN- γ and TNF- α levels in ear homogenates. Treatment with 10 mg/mL of SR suppressed increase of IFN- γ and TNF- α levels significantly. The SR1 and SR10 groups showed almost the same effect on IFN- γ level, with no statistical significance. Treatment with DEX was most effective among the experimental groups (Fig. 4).

Effects of SR on migration of RBL-2H3 cells

SR did not show cytotoxicity up to dose of 400 μ g/mL (Fig. 5A). The cellular migration, induced by 10% FBS, was inhibited by treatment with SR for 24 h in a dose dependent manner up to 100 μ g/mL. Inhibitory effects of > 100 μ g/mL up to 400 μ g/mL of SR were almost the same (Fig. 5B).

Effects of SR on degranulation of RBL-2H3 cells

Because measuring release of β -hexosaminidase and histamine is an indicator of MC degranulation, we examined the anti-allergic activity of SR by measuring release of β -hexosaminidase and histamine from RBL-2H3 cells in vitro. Pretreatment with > 50 μ g/mL of SR lowered the levels of β -hexosaminidase and histamine in a dose-dependent manner. There was no effect evident using 25 μ g/mL SR (Fig. 6).

Discussion

The only etiological treatment of CD is avoidance of the offending agent. In certain circumstances, elimination of the contact allergen is impossible and the therapy is directed at assuaging the inflammatory component (Saint-Mezard et al., 2004; Cohen and Heidary, 2004). Although well-established topical treatments such as corticosteroids and phototherapy exist, they may sometimes be inadvisable or ineffective because of their potential risks or unresponsiveness (Cohen and Heidary, 2004). Recently developed immunomodulators and anti-inflammatory agents have provided various options for treatment of CD (Saint-Mezard et al., 2004; Cohen and Heidary, 2004). In recent years, interest has risen in the use of medicinal plants as complementary and alternative medicines, because of their efficacy, low cost and favorable safety (Wen et al., 2005). In East Asia, many medicinal plants have been used for centuries for the treatment of dermatological disorders such as pruritus, psoriasis and atopic dermatitis. *Rehmanniae Radix*, *Poria Sclerotium*, *Angelica Sinensis Radix*, *Lonicerae Flos* and SR have been frequently used to treat patients with skin disease (Park et al., 2002).

The present study has demonstrated the anti-allergic and anti-inflammatory action of SR in vitro and in vivo. Topical application of SR suppressed allergic reactions in a mouse model of DNFB-induced allergic CD. Increases of ear thickness and weight were prevented by SR (Fig. 2). Inflammatory hyperplasia was also decreased in histopathological examinations (Fig. 3). Spongiosis refers to intercellular epidermal edema, and is recognized as the microscopic hallmark of inflammatory skin disease including allergic contact and nummular dermatitis (Machado-Pinto et al., 1996). Presently, topical application of SR reduced spongiosis and mononuclear cell infiltration effectively (Fig. 3). Taken together, the results indicate that epidermal spongiosis, edema and inflammatory cell infiltration resulted in enlargement of ear thickness and weight, and that SR can effectively prevent these inflammatory reactions in DNFB-induced CD.

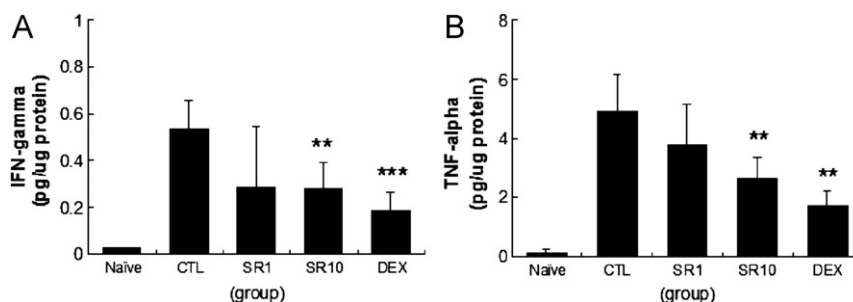


Fig. 4. Effects of SR on levels of IFN- γ and TNF- α in CD mice. Production levels of IFN- γ and TNF- α in ear tissues were measured using the cytometric bead array method. 50 μ g of tissue lysates were used to measure cytokine levels. Naive, non-treated normal mice; CTL, non-treated CD mice; SR1, 1 mg/mL of SR painted CD mice; SR10 10 mg/mL of SR painted CD mice; DEX, 2.5 mg/mL of dexamethasone painted CD mice; (A) IFN- γ ; (B) TNF- α . All values represent as mean $\pm$ SD. * P < 0.01, *** P < 0.001 vs. non-treated CD mice (CTL).

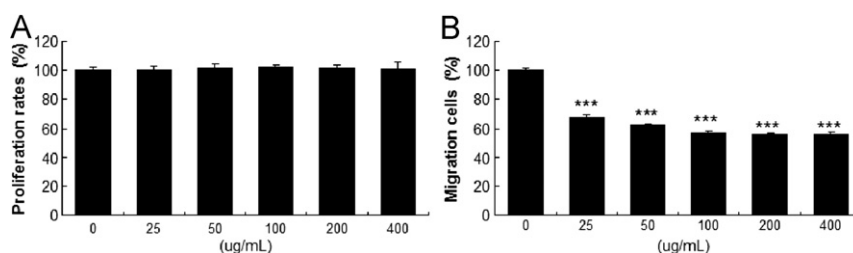


Fig. 5. Effects of SR on proliferation and migration of RBL-2H3 cells. Proliferation rates of RBL-2H3 cells were measured using a modified MTT method. Cells were treated with indicated concentrations of SR for 2 h (A). The transwell migration experiments were performed in a COSTAR 24-well plate as described in materials and methods (B). All values represent mean $\pm$ SD of three independent experiments. * P < 0.05, *** P < 0.001 vs. non-treated control.

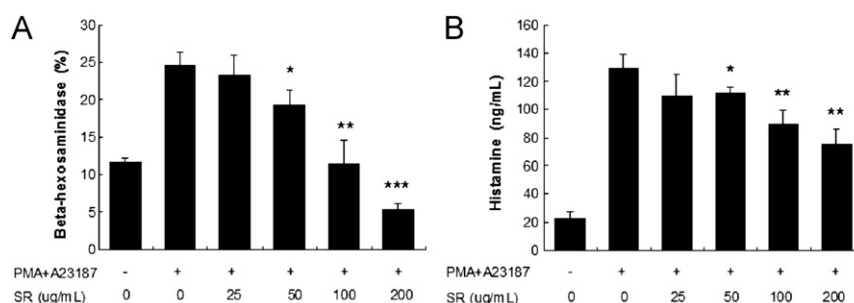


Fig. 6. Effects of SR on degranulation of RBL-2H3 cells. Release of β -hexosaminidase and histamine from RBL-2H3 cells was measured as described in materials and methods. (A) Results for β -hexosaminidase. (B) Results for histamine. All values represent mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. non-treated control.

Skin inflammatory reaction by hapten painting occurs in three steps. First, skin innate immunity is activated. Second, activated T cells produce IFN- γ and cytotoxicity, which results in the activation of skin resident cells and in the production of new mediators of the inflammatory reaction. Third, leukocytes are recruited and progressively induce the morphological changes such as epidermal spongiosis, edema and infiltration of inflammatory cells (Saint-Mezard et al., 2004). IFN- γ , the hallmark of the Th 1 skewing reaction of T cells, is responsible for the increased production in the skin of various cytokines and chemokines such as interleukin-1, TNF- α , granulocyte-macrophage colony-stimulating factor and macrophage inflammatory protein-2, resulting in massive infiltration of leukocytes (Kobayashi, 2008). Prenylated chalcone isolated from SR suppresses the expression of chemokines induced by IFN- γ and TNF- α in human keratinocytes (Choi et al., 2010). Skin contact with the hapten induces the release of TNF- α , which is another Th1 cytokine, and IL-1 β during the sensitization phase (Saint-Mezard et al., 2004). Furthermore, TNF- α exerts a stimulatory effect on skin resident cells, resulting in recruitment of leukocytes during contact hypersensitivity responses (Grabbe and Schwarz, 1998). Presently, SR treatment effectively reduced production levels of IFN- γ and TNF- α in inflammatory tissues (Fig. 4). These data imply that SR is an anti-inflammatory agent against the Th 1 skewing reaction, resulting in reduction of inflammatory reactions such as hyperplasia and spongiosis, and immune cell infiltration.

MCs are secretory cells that play a central role in both the acute and chronic phase of allergic responses (Gould et al., 2003). In many immunological skin diseases including CD, atopic dermatitis and immunobullous disease, MCs increase in number and undergo mediated degranulation (Dvorak et al., 1976; Navi et al., 2007). CHS to specific allergen is primarily due to small-molecule chemical mediators including histamine and β -hexosaminidase from MCs. MCs can affect the function of keratinocytes and fibroblasts by the release of histamine, which can act on keratinocytes and promote their production of adhesion molecules, proinflammatory cytokines and chemokines and growth factors (Kanda and Watanabe, 2004). In addition, MCs and their mediators, especially histamine, may induce activation and proliferation of fibroblasts (Jordana et al., 1988; Hatamochi et al., 1985). Presently, treatment with SR effectively inhibited migration of MCs (Fig. 5) and release of histamine and β -hexosaminidase from RBL-2H3 cells in vitro (Fig. 6). In addition, we confirmed inhibitory effect of SR on MC migration into inflamed tissues using alcian blue staining method in CD mice (data not shown). These data suggest that SR can diminish the infiltration of MCs and release of chemical mediators, resulting in suppression of the inflammatory reaction and hyperplasia.

It is well-known that some intracellular signal pathways are related to activation and degranulation of MCs. SR has been

reported to suppress PMA plus A23187-induced phosphorylation of mitogen-activated protein kinase (MAPK) p38 and c-Jun N-terminal kinase (JNK) in the HMC-1 human mast cell line (Hong et al., 2009). In that report, SR decreases the production of TNF- α , IL-6 and IL-8 in HMC-1 cells in vitro. These results could be clue that suppressed activation of MAPK p38 and JNK by SR might lead to inhibition of degranulation from MCs.

Expression of intercellular cell adhesion molecule (ICAM)-1, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) plays a pivotal role in inflammation. Inhibitions of ICAM-1, iNOS and COX-2 production are considered one of possible mechanisms related to anti-inflammation of SR (Han et al., 2011; Han and Wang, 2012).

Both DEX and SR lowered levels of IFN- γ and TNF- α in this experiment. However, the overall mechanisms of DEX and SR seem to be different from each other. It is well known that corticosteroids suppress overall immune responses (Fedor and Rubinstein, 2006; Kawano et al., 2002) and also reported that corticosteroids can induce weight loss in experimental animals. In certain circumstances weight loss is regarded as one of adverse reactions (Smith et al., 1976). We confirmed that SR did not affect IL-10 levels and body weights. But topical application of DEX lowered IL-10 level in inflamed tissue, and slightly reduced average weight of DEX group (data not shown). These results imply that SR has relatively narrow and selective effects compared to wide range effects of DEX.

Taken together, the data indicate the potential of SR in the treatment of patients with CD and its' potential value for patients who exhibit side effects in response to steroid therapy.

Conclusions

This study was designed to investigate anti-allergic and anti-inflammatory effects of SR using a mouse model of DNFB-induced CD. SR reduced ear swelling, hyperplasia of ear tissue, epidermal spongiosis and prevented infiltration of mononuclear cells and MCs. SR can lower production levels of IFN- γ and TNF- α in inflammatory tissues in vivo. Finally, migration and degranulation of histamine and β -hexosaminidase were inhibited by treatment with SR. These data suggest that SR can be used to treat patients with allergic skin diseases and also suggest that related mechanisms are involved in anti-inflammatory action on the Th 1 skewing reaction and inhibition of the recruitment and degranulation of MCs.

Declaration of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2012.04.053>.

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