Anti-Inflammatory Activity of *Houttuynia cordata* against Lipoteichoic Acid-Induced Inflammation in Human Dermal Fibroblasts

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Houttuynia cordata (*H. cordata*) has anti-inflammatory and anti-bacterial activities. *Staphylococcus aureus* (*S. aureus*)-producing lipoteichoic acid (LTA) has been implicated in inflammatory and immunologic responses in patients with atopic dermatitis (AD). The purpose of this study was to determine the potential of *H. cordata* as a novel anti-inflammatory agent for AD by evaluating the anti-inflammatory activity of *H. cordata* water extract on LTA-induced inflammation in dermal fibroblasts (DFs). The anti-inflammatory activity of *H. cordata* was evaluated by studying the inflammatory markers tumor necrosis factor-α (TNF-α) and cyclooxygenase-2 (COX-2). As shown by MTT assay and COX-2 immunoblot, *H. cordata* was not toxic to DFs in doses up to 20 μg/ml. In RT-PCR and Western blot analysis, LTA up-regulated TNF-α (mRNA) and COX-2 (protein) at doses of 5~20 μg/ml in a dose-dependent manner. Next, DFs were treated with LTA (10 μg/ml) in the absence (control) or presence of *H. cordata* for 24 h. The level of LTA-induced TNF-α mRNA was suppressed by *H. cordata* (20 μg/ml) by up to 40% compared with the level in LTA-treated control samples. Under the same conditions, the level of LTA-induced COX-2 protein was suppressed by *H. cordata* treatment by up to 52% compared with the level in LTA-treated control samples. When DFs were treated with TNF-α (10 μg/ml) for 24 h, TNF-α-induced COX-2 expression was down-regulated by *H. cordata* treatment to 65% of that in control samples. In conclusion, *H. cordata* has anti-inflammatory activity against LTA-induced inflammation in DFs, in part by blocking the TNF-α pathway.

Key Words: *Plant extract, Houttuynia cordata; Lipoteichoic acid; Tumor necrosis factor-alpha; Cyclo-oxygenase 2*

Introduction

Houttuynia cordata (*H. cordata*) is a traditional medicinal plant that is used in herbal preparations in Asian countries to relieve inflammatory conditions. *H. cordata* has been shown to have strong anti-inflammatory activity in a pleurisy model and in ear edema.[1] *H. cordata* has been used as an anti-inflammatory agent for treating atopic dermatitis (AD) and acne in traditional Korean herbal medicine.

With respect to the mechanism by which AD inflammation is induced, *Staphylococcus aureus* has been widely studied for its active roles in inflammatory and immunologic responses against AD.[2] Lipoteichoic acid (LTA) acts as an adhesion molecule for many Gram-positive bacteria, including staphylococci, streptococci,

and pneumococci.[3] Recently, LTA was extensively studied in relation to the pathogenic role of *S. aureus* in AD inflammation.[4]

S. aureus cell walls are composed of highly cross-linked peptidoglycans (PEGs) decorated with teichoic acid (TA) polymers, which are also linked to plasma membrane phospholipids, of which LTA and PEG are the major surface components.[5] TA or related glyco-polymers play crucial roles in bacterial survival under suboptimal conditions and in other basic cellular processes.[3,4] In AD, LTA induces IL-5 production from peripheral blood mononuclear cells (PBMCs) and AD-like skin lesions with the T helper type 2 (Th2) response, including the production of IL-4 and IL-5.[5]

Tumor necrosis factor (TNF)-α has a number of pro-inflammatory effects in skin inflammation and therefore has been studied extensively as a therapeutic target in treating TNF-α-mediated skin disorders, such as AD and psoriasis.[6] In skin affected by AD, local tissue expression of pro-inflammatory cytokines and chemokines has focused on epidermal keratinocytes (KCs), T lymphocytes, dendritic cells, macrophages, mast cells, and eosinophils.[7] *S. aureus* induces the production of major pro-inflammatory cytokines, including TNF-α, interleukin-1 (IL-1), IL-6, and IL-8 from KCs in the epidermis or from PBMCs, which are infiltrated into the dermis.[8,9]

However, dermal fibroblasts (DFs) have been reported to be another source of TNF-α production by various external stimuli.[10-12] In this study, we determined whether DFs can be another source of production following stimulation by LTA. COX-2 is also a good biomarker for inflammation in various tissues, including skin, as it has been widely studied in relation to tissue inflammation, wounds or ulcer healing, and carcinogenesis.[13] In this study, we used TNF-α and COX-2 as representative inflammatory markers.

The purpose of the present study was to evaluate the anti-inflammatory activity of water extract of *H. cordata* against LTA-induced inflammation in DFs, with the prospect of developing a novel anti-inflammatory agent for AD in the future.

Materials and Methods

1. Preparation of water extract of *H. cordata* leaves

H. cordata leaves were collected from the Gwangju area in South Korea. The water extract was obtained by boiling 100 g of *H. cordata* leaves in 1 L of distilled water for 1 h. The extract was filtered and was concentrated by lyophilization. The extract yield from the fresh leaves was approximately 4.5%. *H. cordata* water extract powder was stored at $-20^{\circ}C$ until used in the experiments.

2. Primary culture of DFs

Intact human skin from circumcised foreskin was obtained after informed consent according to the guidelines of the local Ethics Committee of Chonnam National University Hospital Institutional Review Board and the Declaration of Helsinki Principles. The skin was cut into small pieces and was then treated with 2.4 U/ml dispase (Boehringer Mannheim, Ingelheim, Germany) for 30 min at $37^{\circ}C$. The dermal tissues were separated from the epidermis and minced into small pieces with iris scissors, and primary DFs were obtained by culturing the tissue in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 incubator. DFs from passages 2 to 4 were used for all experiments. The primary cultured cells were confirmed to be DFs by immunostaining with vimentin (Fig. 1).

3. MTT assay

To determine cell viability, the cells were seeded into 96-well plates and treated with *H. cordata* extracts for 24 h. We used a colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) kit (Chemicon International, Inc., Billerica, MA, USA) for determining cell survival and proliferation, according to the manufacturer's instructions. Briefly, 10 ml of MTT
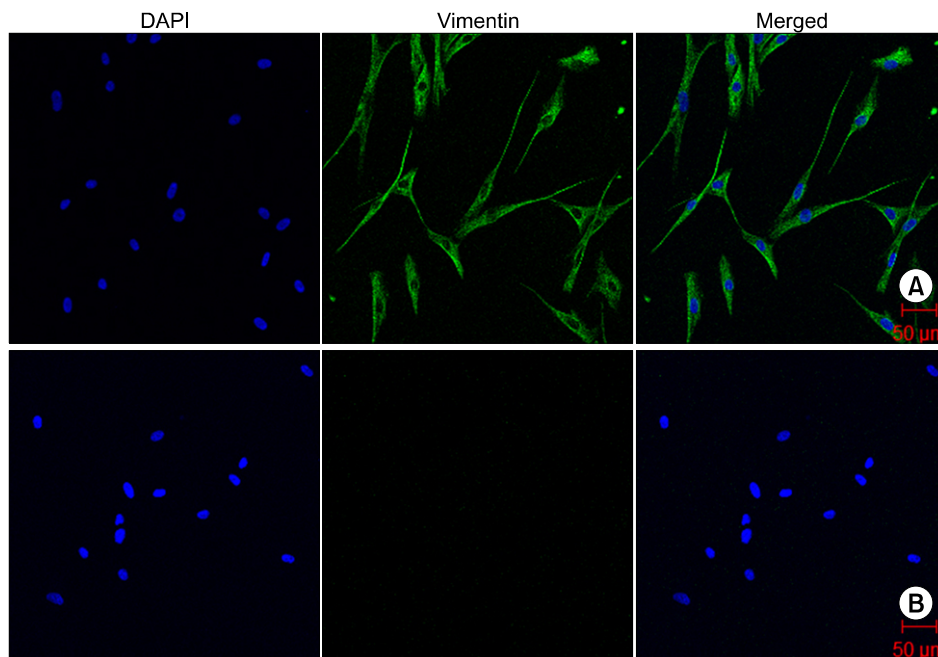
Fig. 1. Immunocytochemical staining for vimentin in DFs. (A) Confocal laser scanning microscopy demonstrated positive fluorescence signals for vimentin (green). Nuclei were stained with DAPI (blue). (B) Negative controls stained only with secondary antibody. Scale bar: 50 μm.

solution was added to each well (0.5 mg/ml), and the plates were incubated at 37°C for 2 h. The resulting formazan crystals were dissolved with 100 ml of dimethylsulfoxide (DMSO) and absorbance was read at 570 nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). In this study, LTA from *S. aureus* was purchased from Sigma Chemical Company (St. Louis, MO, USA). From reviewing published reports,[14,15] the optimal concentration of LTA was determined to be 10 μg/ml; no cytotoxicity was demonstrated on the basis of the MTT assay (data not shown).

4. Western blot analysis

Whole-cell extracts were prepared by sonication in buffer (1% Triton X-100, 20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5% glycerol, 1 mM EDTA [pH 8.0], and protease inhibitor cocktail). After centrifugation (12,000 × g, 15 min at 4°C), the protein concentration of the supernatant was measured by using a Coomassie plus protein assay reagent kit (Pierce, Rockford, IL, USA). The cell extract (30 μg) in SDS sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 5% β-mercaptoethanol)

was separated by 10% SDS-PAGE and transferred onto a PVDF membrane (0.45 μm; Millipore, Billerica, MA, USA). After incubation with blocking solution (5% skim milk in Tris-buffered saline with 0.1% Tween-20 [TBS-T]) for 1 h at room temperature, membrane-bound proteins were probed overnight with anti-COX-2 (ab15191, 1 : 1,000; Abcam, Cambridgeshire, UK) and mouse monoclonal β-actin antibodies (ab-6276, 1 : 5,000; Abcam), followed by incubation with HRP-conjugated goat anti-rabbit IgG (1 : 5,000) or anti-mouse IgG (1 : 5,000). Antibody-bound protein bands were visualized by enhanced chemiluminescence detection. Densitometric analysis for COX-2 expression was based on three independent experiments.

5. Semi-quantitative RT-PCR

Total RNA was isolated by using an RNeasy mini-kit (Qiagen, Fremont, CA, USA) according to the manufacturer's instructions. cDNA was generated from 1 μg of total RNA by using the Omniscript RT kit (Qiagen). For semi-quantitative PCR, a PCR pre-mixture kit (ELPIS, Taejeon, Korea) was used. PCR reactions for TNF-α and GAPDH were performed by using methods described before.[12] PCR products were analyzed using 1.5% agarose

gel electrophoresis, stained with SYBR safe gel stain buffer (Invitrogen, Carlsbad, CA, USA), and visualized by luminescence (LAS 3000, Fujifilm, Tokyo, Japan). Triplicate experiments were performed for the same condition, and the relative intensity for positive bands was analyzed by use of a densitometer.

6. Immunocytochemical staining for vimentin

Immunocytochemistry for confocal microscopy was performed on vimentin. DFs were seeded in each well of the slide chamber at a density of 3×10^3 cells per well. After washing with PBS, cells were fixed in 4% paraformaldehyde in PBS for 10 min and were then permeabilized with PBS containing 0.5% Triton X-100 (PBS-T) for 15 min. The cells were incubated with PBS-T containing 1% BSA for 1 h and stained with anti-vimentin antibody (M0725, 1 : 300; DakoCytomation, Carpenteria, CA, USA) overnight at 4°C, followed by incubation with Alexa Fluor 488 chicken anti-mouse IgG (A21200, 1 : 500; Invitrogen) for 1 h. Images were visualized by confocal microscopy with a 20X objective on a laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany) and were analyzed by using the LSM 5 browser imaging software.

7. Statistics

All experiments were carried out in triplicate, and the results are expressed as the mean ± standard deviation. Comparisons between samples were carried out by using Student's t-test with statistical significance considered to be $p < 0.05$.

Results

1. LTA induces up-regulation of TNF-α and COX-2 in DFs

As a preliminary experiment, we evaluated the optimal concentration of an *H. cordata* extract. As shown by the MTT assay, the viability of the DFs was not significantly affected by *H. cordata* at doses of $1 \sim 100 \, \mu$g/ml (Fig.

2A). However, COX-2 protein expression was stimulated by the highest dose of *H. cordata* at 50 μg/ml (Fig. 2B), implying a toxic response of DFs to *H. cordata*. There was no effect of *H. cordata* treatment on TNF-α expression (Fig. 2C). Thus, the next experiments with *H. cordata* were performed at concentrations up to 20 μg/ml. Among the pro-inflammatory molecules involved in the
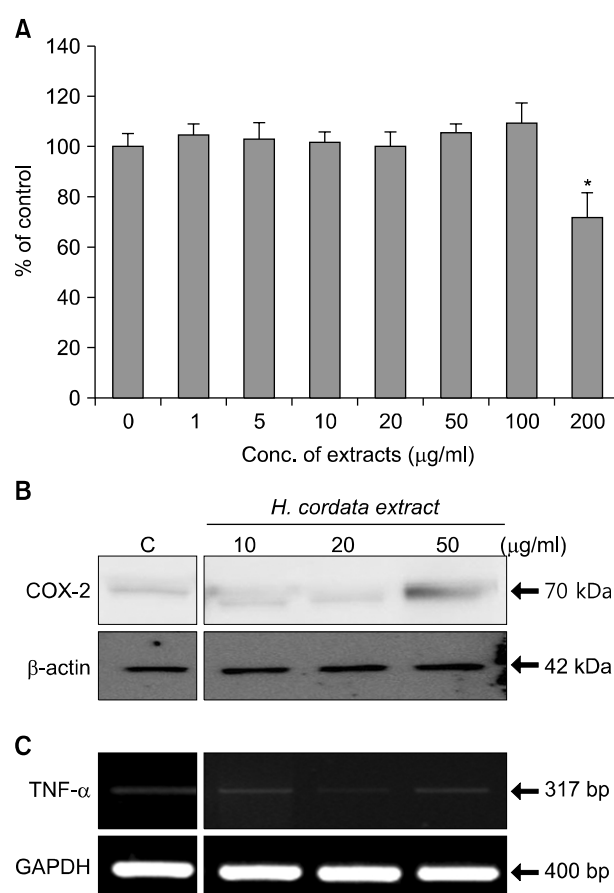

Fig. 2. Cell viability assay and levels of imflammatory markers to determine optimal concentrations of *H. cordata* and LTA in DFs. (A) DFs were treated with *H. cordata* water extracts at different concentrations (1 μg/ml to 200 μg/ml), and cell viability was measured with an MTT assay kit. The cell viability of control samples, which were not treated with *H. cordata* extract, was designated as 100%. The data represent the mean viability values of six samples that were treated with the same concentration of *H. cordata* extract. (B) Western blot analysis for the level of COX-2 expression in DFs, which were treated with *H. cordata* extract at different concentrations (10~50 μg/ml). C (control) indicates the level of COX-2 protein in DFs that were not treated with *H. cordata* extract. (C) RT-PCR for TNF-α mRNA expression was performed with samples that were harvested 24 h after *H. cordata* treatment (10~50 μg/ml). For an internal standard, RT-PCR for the *GAPDH* gene was performed with each sample. *p < 0.05.
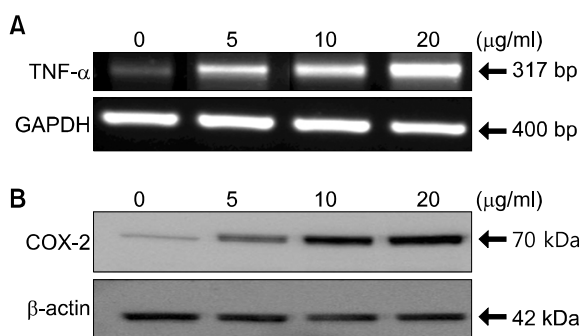
Fig. 3. Dose-dependent expression of TNF-α and COX-2 by LTA in DFs. (A) DFs were treated with LTA at concentrations of 5, 10, and 20 μg/ml, and RT-PCR for TNF-α mRNA expression was performed with samples that were harvested 24 h after LTA treatment. For an internal standard, RT-PCR for the *GAPDH* gene was performed with each sample. (B) Under the same conditions, protein levels of COX-2 were checked by Western blot analysis. For an internal standard, the β-actin protein level of each sample was measured. This picture is representative of three experiments.
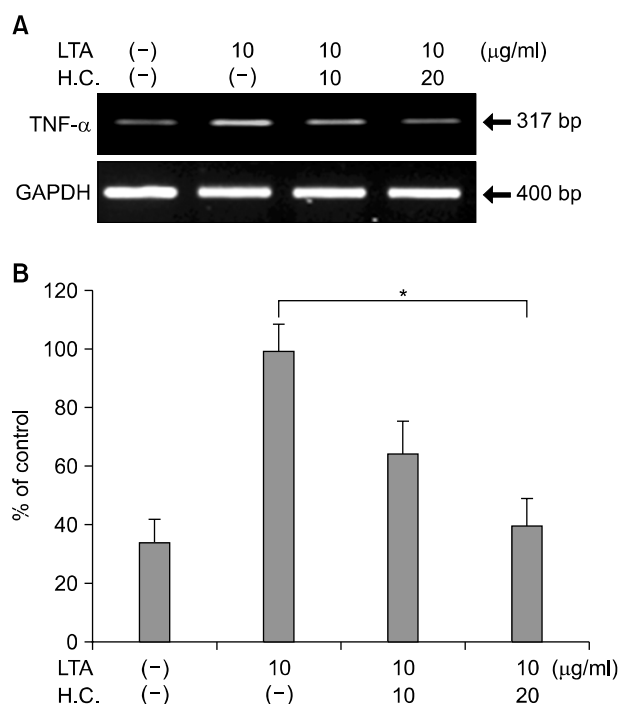

Fig. 4. Suppression of LTA-induced TNF-α expression by *H. cordata* extract in DFs. (A) DFs were treated with *H. cordata* extract (H.C.) at concentrations of 10 and 20 μg/ml in the presence of LTA (10 μg/ml), and RT-PCR for TNF-α mRNA expression was performed. This picture is representative of three experiments. (B) Densitometric analysis for TNF-α mRNA levels from triplicate experiments was performed, and the relative density of each sample was depicted compared to the highest level (100%) of the sample, which was only treated with LTA (10 μg/ml). *p<0.05 for significant differences between the groups (n=3).

pathogenesis of AD inflammation, TNF-α was shown to be a major cytokine in inducing AD inflammation.[6] In DFs treated with LTA (10 μg/ml) for 24 h, the level of TNF-α mRNA based on RT-PCR was up-regulated in a dose-dependent manner (Fig. 3A). As shown by Western blot analysis, the level of COX-2 protein was up-regulated with a positive correlation between the LTA dose and COX-2 level (Fig. 3B).

2. *H. cordata* inhibits LTA-induced TNF-α and COX-2 expression in DFs

We next evaluated whether *H. cordata* had anti-inflammatory activity in LTA-induced inflammation in DFs. When DFs were treated with *H. cordata* extract at doses of 20 μg/ml for 24 h, the level of LTA (10 μg/ml)-induced TNF-α mRNA was reduced to approximately 40% of LTA-treated control samples (p<0.05, n=3) (Fig. 4). Under the same conditions, the level of LTA-induced COX-2 protein was reduced to 52% of that in LTA-treated control samples (p<0.05, n=3) (Fig. 5).

3. *H. cordata* inhibits TNF-α-induced COX-2 expression in DFs

From the results that LTA could induce TNF-α mRNA expression, we evaluated whether *H. cordata* had anti-inflammatory activity in TNF-α-treated DFs. When DFs were treated with *H. cordata* extract at doses of 20 μg/ml for 24 h, TNF-α-induced COX-2 expression was reduced to 65% of that in TNF-α-treated control samples (p<0.05, n=3) (Fig. 6).

Discussion

H. cordata is a native herbaceous plant in many Asian countries. *H. cordata* has been used to treat various infectious conditions owing to its anti-viral activity against herpes virus type 1, influenza virus, and human immunodeficiency[16] and its antibacterial activity against *S. aureus, Sarcina ureae,*[17] *Propiniobacterium acnes*

A

| LTA | (−) | 10 | 10 | 10 | (μg/ml) |
| H.C. | (−) | (−) | 10 | 20 | |

COX-2 ← 70 kDa

β-actin ← 42 kDa

B

A

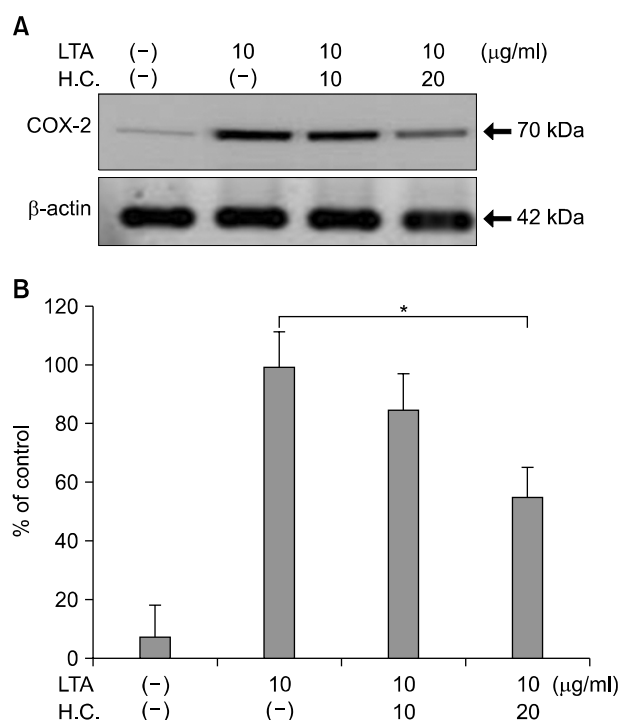| TNF-α | (−) | 10 | 10 | 10 | (μg/ml) |
| H.C. | (−) | (−) | 10 | 20 | |

COX-2 ← 70 kDa

β-actin ← 42 kDa

B

Fig. 5. Suppression of LTA-induced COX-2 expression by *H. cordata* extract in DFs. (A) DFs were treated with *H. cordata* extract (H.C.) at concentrations of 10 and 20 μg/ml in the presence of LTA (10 μg/ml), and Western blot analysis for COX-2 protein expression was performed. (B) Densitometric analysis for COX-2 protein levels from triplicate experiments was performed, and the relative density of each sample was depicted compared to the highest level (100%) of the sample, which was treated only with LTA (10 μg/ml). *p < 0.05 for significant differences between the groups (n=3).
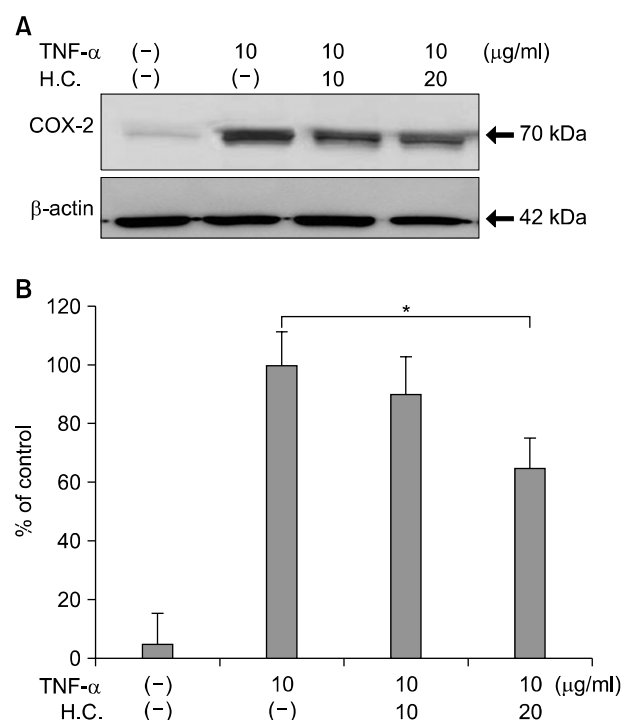
Fig. 6. Suppression of TNF-α-induced COX-2 expression by *H. cordata* extract in DFs. (A) DFs were treated with *H. cordata* extract (H.C.) at concentrations of 10 and 20 μg/ml in the presence of TNF-α (10 μg/ml), and Western blot analysis for COX-2 protein expression was performed. (B) Densitometric analysis for COX-2 protein levels from triplicate experiments was performed, and the relative density of each sample was depicted compared to the highest level (100%) of the sample, which was treated only with TNF-α (10 μg/ml). *p < 0.05 for significant differences between the groups.

(*P. acnes*),[18] and *Salmonella typhimurium*.[19] *H. cordata* also has other biological activities, such as anti-inflammatory,[19] anti-cancer,[20] and anti-oxidant activities.[21] Some components of *H. cordata*, including methyl nonyl ketone, caryophyllene, bornyl acetate, α-pinene, β-pinene, and limonene, have been reported to have antibacterial properties against the Gram-positive bacteria *S. aureus* and *Sarcina ureae*.[17] With respect to antioxidant activity, other components of *H. cordata*, including quercitrin, quercetin-3-O-β-D-galactopyranose, and protocatechuic acid, were isolated from *H. cordata* extract.[21] AD is a chronically relapsing skin disease manifesting with recurrent attacks of eczematous inflammation in the skin. AD develops from complex interactions between genetic alteration and environmental agents. Among several proposed environmental

factors, *S. aureus* is extensively studied in relation to the pathogenesis of AD, because the microorganism was revealed to be a major pathogen for inducing inflammatory and immunologic responses in the skin of AD patients. The altered skin structure or barrier function and a deficiency of antimicrobial peptides favor colonization of *S. aureus* in the skin of patients with AD, because their endotoxins with superantigenic activity stimulate the activation of T cells or other cells involved in immunologic responses.[22] Taken together, *H. cordata* might be a candidate therapeutic agent to treat AD by controlling inflammatory or oxidative responses as well as *S. aureus* infections in the skin of patients with AD.

The results of this study showed that *H. cordata* has a strong anti-inflammatory activity against *S. aureus*

LTA-induced inflammatory responses, partly through inhibiting TNF-α expression in DFs. In this study, *H. cordata* extract did not affect the expression of COX-2 or TNF-α at doses of $10 \sim 20 \mu$g/ml, but *H. cordata* extract at 50μg/ml up-regulated COX-2 expression, suggesting a toxic response of DFs at the highest dose of *H. cordata*. Among the cellular components of skin, KCs are known to be the major cells that produce TNF-α, and DFs serve as a target of TNF-α that is secreted from KCs or inflammatory cells, including lymphocytes, monocytes, mast cells, and macrophages.[23] *S. aureus* LTA can efficiently stimulate human T cells and monocytes to produce TNF-α via Toll-like receptor-2.[24] TNF-α has myriad pro-inflammatory effects on skin inflammation, but TNF-α is barely detectable from KCs or DFs of the skin under normal conditions. However, ultraviolet (UV)-B irradiation induces TNF-α from KCs and DFs,[10,11] implying that UV radiation or proinflammatory stimuli induce the production of TNF-α from both cells. In agreement with our previous report that *P. acnes* induced TNF-α production from DFs,[12] this study showed that LTA also stimulates the production of TNF-α from DFs. Because the inflammatory reaction of AD takes place in the dermis rather than the epidermis, our results suggest that DFs are not only a target of TNF-α that is produced by other cells (KCs and inflammatory cells) but also a source for TNF-α expression by LTA stimulation. When DFs were treated with TNF-α, *H. cordata* had an inhibitory effect against TNF-α-induced COX-2 expression.

Among the three isoforms of COX, COX-2 is widely studied owing to its diverse physiologic and pathologic processes, including inflammation. COX-2 is also induced in response to inflammatory stimuli, such as UV radiation, lipopolysaccharide (LPS), and 12-O-tetradecanoylphorbol 13-acetate (TPA) or phorbol ester in the skin.[13,25-27] In this study, we used TNF-α and COX-2 as inflammatory markers of DFs.

The cytokine production by LTA is similar to that induced by whole bacteria.[28] LTA from *S. aureus* can promote the induction of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 from monocytes or macrophages,[29] which was also induced by whole bacteria. In this study, we found that LTA induces an inflammatory response by stimulating TNF-α production as well as COX-2 in DFs. Taken together, our results showing that *H. cordata* has inhibitory activity against LTA-induced inflammation in DFs suggest that *H. cordata* might be a novel anti-inflammatory agent for relieving AD inflammation. The present study supports the traditional medicine practice in Korea in which *H. cordata* is used as an anti-inflammatory agent to relieve inflammatory conditions, such as acne and AD. Further studies should be performed to clarify which components of *H. cordata* have anti-inflammatory activity in LTA-induced inflammation in DFs. Our *in vitro* results should be validated by *in vivo* experiments in animals and humans in the future.

Acknowledgement

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