Methyl 5-Chloro-4,5-didehydrojasmonate (J7) Inhibits Macrophage-Derived Chemokine Production *via* Down-Regulation of the Signal Transducers and Activators of Transcription 1 Pathway in HaCaT Human Keratinocytes

Gyeong-Jin Kang,^a Hung-The Dang,^b Sang-Chul Han,^a Na-Jin Kang,^c Dong-Hwan Koo,^c Young Sang Koh,^{a,c} Jin Won Hyun,^{a,c} Hee-Kyoung Kang,^{a,c} Jee H. Jung,^b and Eun-Sook Yoo^{*,a,c}

^aDepartment of Medicine, Jeju National University School of Medicine; ^cDepartment of Biomedicine & Drug Development, Jeju National University; Jeju 690–756, Republic of Korea; and ^bCollege of Pharmacy, Pusan National University; Busan 609–735, Republic of Korea.

Received February 18, 2013; accepted July 19, 2013

Jasmonates are lipid-based stress hormones that are critical for the defense of plants against insects. Two naturally occurring jasmonates, jasmonic acid and methyl jasmonate, have recently been explored for their efficacy as anti-cancer agents. Furthermore, certain synthetic jasmonates (*e.g.*, the cyclopentenone isoprostane J2) exert anti-inflammatory actions in lipopolysaccharide (LPS)-challenged murine macrophages *via* down-regulation of chemokines and other inflammatory mediators. Chemokines participate in the development and progression of many inflammatory disorders, such as atopic dermatitis (AD) and Crohn's disease, as exemplified by the role of macrophage-derived chemokine (MDC/CCL22) in the pathology of AD. The current study therefore investigated the impact of jasmonate derivatives (jasmonic acid and methyl jasmonate) and their synthetic analogues (J2 and J7) on the expression of MDC in interferon (IFN)- γ - and tumor necrosis factor (TNF)- α -stimulated HaCaT human keratinocytes, as well as the attendant mechanism of action. Jasmonic acid, methyl jasmonate, and J2 failed to inhibit the cytokine-stimulated production of MDC. By contrast, J7 suppressed the mRNA and protein expression levels of MDC in a dose-dependent manner. Moreover, J7 diminished the activation of signal transducers and activators of transcription 1 (STAT1), but had no inhibitory effect on the nuclear factor kappa B (NF- κ B) or mitogen-activated protein kinase (MAPK) pathways. These results demonstrate that J7 impairs IFN- γ - and TNF- α -induced inflammatory chemokine production by targeting the STAT1 pathway.

Key words jasmonate; signal transducers and activators of transcription 1; HaCaT keratinocyte

Jasmonic acid and its methyl ester, methyl jasmonate, are fatty acid-derived cyclopentanones that are synthesized from linolenic acid residing in the chloroplast membrane. These lipid-based stress hormones occur throughout the plant kingdom and play major roles in the defense of plants against insects and disease. Many reports suggest that jasmonates can also suppress the proliferation of various cancer cells, leading to the death of tumorigenic but not normal cells.^{1–3)}

Jasmonic acid and methyl jasmonate represent typical active jasmonate derivatives. Interestingly, their chemical makeup shares the same partial structure with anti-inflammatory prostaglandins. We recently selected methyl jasmonate as the starting material for the synthesis of cyclopentenone prostaglandin-like compounds, termed isoprostanes, and reported that several synthetic jasmonates based on the structure of methyl jasmonate showed inhibitory effects on inflammatory mediators in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. In particular, methyl 4,5-didehydrojasmonate (the J2 compound) demonstrated strong anti-inflammatory activity *via* regulation of the nuclear factor kappa B (NF- κ B) pathway. Furthermore, a new synthetic methyl dehydrojasmonate compound, methyl 5-chloro-4,5-didehydrojasmonate (J7), also showed inhibitory activity against LPS-induced inflammatory mediators in murine macrophages.^{4–6)} However, the ability of jasmonic acid, methyl jasmonate, J2, and J7 to regulate the expression of mediators associated with inflammatory skin

conditions has not yet been assessed in keratinocytes.

As such, the present study delineated the impact of these jasmonate derivatives and their synthetic analogues on the production of a key atopic dermatitis (AD)-related chemokine, macrophage-derived chemokine (MDC/CCL22). We also investigated the mechanism of action of jasmonic acid, methyl jasmonate, J2, and J7 in interferon (IFN)- γ - and tumor necrosis factor (TNF)- α -stimulated HaCaT human keratinocytes.

Results and Discussion

The structure of the four compounds employed in this study (jasmonic acid, methyl jasmonate, methyl 4,5-didehydrojasmonate (J2), and methyl 5-chloro-4,5-didehydrojasmonate (J7)) are shown in Fig. 1.

The HaCaT cell line is immortalized and has a transformed phenotype *in vitro*, but maintains full epidermal differentiation capacity.⁷⁾ Furthermore, this cell line expresses various cytokines and chemokines that are involved in the development of inflammatory skin diseases under appropriate stimulatory conditions.^{8–10)} Therefore, HaCaT cells are a useful substitute for primary human keratinocytes for the screening of pharmacologically active plant extracts and their constituent chemicals, as well as elucidation of their associated mechanism(s) of action.

We first investigated the effects of jasmonic acid, methyl jasmonate, J2, and J7 on inflammatory chemokine production in IFN- γ (10 ng/mL)- and TNF- α (10 ng/mL)-stimulated HaCaT keratinocytes. Chemokines are small proteins (67 to

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: eunsyoo@jejunu.ac.kr

Fig. 1. Chemical Structures of Jasmonic Acid, Methyl Jasmonate, Methyl 4,5-Didehydrojasmonate (J2), and Methyl 5-Chloro-4,5-didehydrojasmonate (J7)

127 amino acids) released from various cell types that regulate the infiltration of immune cells to inflammatory or infectious sites. Furthermore, several inflammatory cytokines stimulate the expression of chemokines, especially those chemokines that are enhanced in the serum of AD patients.^{8,11–14} For example, the ligand for CC chemokine receptor 4 (CCR4), MDC/CCL22, is mainly expressed in T helper 2 (Th2) lymphocytes, basophils, and natural killer cells and is the most characteristic inflammatory chemokine in AD. This in turn suggests that MDC/CCL22 produced by keratinocytes could be a key molecule in attracting inflammatory lymphocytes to the skin.^{15–19} In this study, we confirmed that single treatment of IFN- γ induced the MDC production in HaCaT cells and it increased by co-treatment of IFN- γ and TNF- α (Fig. S1). Hence, we examined the activity of synthetic jasmonates in the condition of co-treatment of IFN- γ and TNF- α .

Jasmonic acid and methyl jasmonate showed no effect on the viability of HaCaT keratinocytes. Furthermore, although IFN- γ and TNF- α stimulation clearly induced the production of MDC in HaCaT cells, treatment with jasmonic acid or methyl jasmonate had no impact on the production of MDC compared with IFN- γ and TNF- α alone (Fig. 2). Interestingly, J2 also failed to inhibit the cytokine-stimulated production of MDC (Fig. 3). By contrast, treatment with J7 significantly suppressed the induction of MDC by IFN- γ and TNF- α in a concentration-dependent manner (Figs. 3, 4). Additionally, the level of MDC mRNA was augmented after stimulation with cytokines for 6h, but pre-treatment with J7 dramatically suppressed the expression of MDC mRNA in a dose-dependent manner (2.5, 5, 10 μ M) (Fig. 5). In the cell viability assay, J7 actually increased the vitality of HaCaT keratinocytes relative to the control (Figs. 3, 4, S2). This result indicates that J7 can modulate the expression of an inflammatory chemokine, MDC, in IFN- γ - and TNF- α -stimulated HaCaT keratinocytes. Based on the current observations, J7, as opposed to J2, jasmonic acid, or methyl jasmonate, can potentially inhibit MDC production and might function through an alternative signaling pathway relative to the other three compounds.

The promoter region of MDC contains binding sequences

for signal transducers and activators of transcription (STAT), NF- κ B, and activating protein (AP)-1, and these transcription factors may mediate the transcription of the MDC gene.^{20–22} IFN- γ acts *via* IFNGR1 and IFNGR2 receptor subunits, which form a heterotetramer on the cell surface, and activates various signaling cascades. In particular, the Janus kinase (Jak)-STAT pathway is a critical signaling pathway stimulated by IFN- γ that consists of the non-receptor tyrosine kinases Jak1 and Jak2, as well as STAT.

Phosphorylated STAT1 functions as a transcription factor that activates primary response genes related to inflammatory processes.^{23,24} Furthermore, several plant extracts and compounds have been shown to inhibit the actions of inflammatory chemokines *via* the regulation of signaling pathways stimulated by IFN- γ and TNF- α , including STAT1, NF- κ B, and mitogen-activated protein kinase (MAPK) cascades.^{25–28} Among these transcription factors, the STAT1 protein is a crucial and specific regulator of IFN- γ -induced signals that controls the transcription of target genes, including MDC.^{23,29} Therefore, we tested the effect of J7 on the activation of STAT1 in IFN- γ - and TNF- α -treated HaCaT keratinocytes and detected a high level of phosphorylated STAT1 at 15 min after cytokine treatment. Conversely, pre-treatment of the cells with J7 dramatically and dose-dependently suppressed STAT1 phosphorylation (Fig. 6A). In addition, microscopy results showed that stimulation of HaCaT keratinocytes with IFN- γ and TNF- α led to nuclear translocation of STAT1 within 1h. However, the nuclear translocation was effectively repressed by pre-treatment of the cells with J7 (Fig. 6B).

Last, we confirmed the effect of J7 on the NF- κ B and MAPK signaling pathways in IFN- γ - and TNF- α -treated HaCaT keratinocytes. Various studies have reported that several stimuli, including IFN- γ and TNF- α , can also activate the extracellular signal-regulated kinase (ERK), p38 MAPK, and NF- κ B pathways.^{30–32} Under normal conditions, NF- κ B combines with inhibitor of kappa B- α (I κ B- α) in the cytoplasm. IFN- γ and TNF- α , among other cytokines, can induce the phosphorylation and degradation of I κ B- α , leading to the subsequent phosphorylation of NF- κ B and its translocation

Fig. 2. Effects of Jasmonic Acid and Methyl Jasmonate on MDC Production in IFN- γ - and TNF- α -Stimulated HaCaT Keratinocytes

HaCaT cells (2.0×10^5 cells/mL) were pre-incubated in unsupplemented medium for 18 h. MDC production was then determined in the supernatants of cells stimulated with IFN- γ (10 ng/mL) and TNF- α (10 ng/mL) in the presence of (A) jasmonic acid (JA) or (B) methyl jasmonate (MJ) at the indicated concentrations for 24 h. MDC production was measured by ELISA, and cell viability was determined by the WST assay. The measurements of MDC were done in triplicate. Bars and error bars indicate the mean $\pm$ the S.D.

into the nucleus. Whereas I κ B- α is highly expressed in unstimulated HaCaT cells, we observed its maximal degradation 15 min after the addition of IFN- γ and TNF- α . Furthermore, the nuclear translocation of NF- κ B increased at 60 min after the initiation of cytokine treatment. However, pre-treatment of the cells with J7 did not inhibit these features of the NF- κ B signaling pathway (Figs. 7A,B). Some reports show that compounds or extract from natural product decrease the MDC production *via* inhibition of NF- κ B and STAT1 activation.^{33,34} Even though STAT1 and NF- κ B signal were activated by IFN- γ and TNF- α in our condition, J7 only inhibited the activation of STAT1 signal. In RAW264.7 macrophages, J7 also inhibited the STAT1 activation, but not NF- κ B, by LPS stimulation (Fig. S4). Interestingly, addition of PDTC, NF- κ B signal inhibitor, failed to reduce the MDC expression by single treatment of IFN- γ (Fig. S5). Furthermore, we have been reported that J2 inhibits the inflammatory mediators by LPS *via* the inhibition of NF- κ B signal.⁵ However, as shown in Fig. 3, J2 showed no effect on MDC production by IFN- γ and TNF- α . Together with Fig. S1, these results indicated that NF- κ B sig-

nal may partly be involved in MDC production by IFN- γ and/or TNF- α and that J7 may specifically act on STAT1 pathway than other pathways.

The MAPK pathway is reportedly involved in the production of inflammatory chemokines and was also activated in our study by exposure of HaCaT cells to IFN- γ and TNF- α . However, all three MAPK pathways (*i.e.*, ERK, anti-phospho-c-Jun N-terminal kinase (JNK), and p38 MAPK signaling cascades) were further up-regulated by treatment with J7 (Fig. 7C). Interestingly, this result may mean that the increased cell viability on HaCaT and RAW264.7 cells is related with ERK activation by J7 (Figs. 3, 5, S2, S3) because MAPK pathways, especially ERK signal, are known to control a cell proliferation. Although additional experiments are required to elucidate whether the activation of a specific MAPK is involved in the ability of J7 to decrease MDC production, based on the current results, we anticipate that the inhibitory effect of J7 is mediated by down-regulation of STAT1 transcriptional activity rather than by activation of a MAPK pathway.

Fig. 3. Effect of J7 on MDC Production in IFN- γ - and TNF- α -Stimulated HaCaT Keratinocytes

HaCaT cells (2.0×10^5 cells/mL) were pre-incubated in unsupplemented medium for 18 h. MDC production was then determined in the supernatants of cells stimulated with IFN- γ (10 ng/mL) and TNF- α (10 ng/mL) in the presence or absence of J7 at the indicated concentrations for 24 h. MDC production was measured by ELISA, and cell viability was determined by the WST assay. The measurements of MDC were done in triplicate. Bars and error bars indicate the mean $\pm$ S.D. ** $p < 0.01$, *** $p < 0.001$, significant differences compared with the positive control.

Conclusion

In conclusion, among the compounds tested in this study, only J7 strongly suppressed MDC production in HaCaT human keratinocytes. Furthermore, J7 specifically inhibited the activation of STAT1 induced by IFN- γ and TNF- α . These data provide new evidence regarding the anti-chemokine function and the mechanism of action of a new analogue that has the basic structure of jasmonate. Given the comparatively limited amounts of naturally occurring jasmonates, J7 could be an appropriate substitute for pharmacological use in the treatment of AD.

Experimental

Chemicals and Reagents Jasmonic acid and methyl jasmonate were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Methyl 4,5-didehydrojasmonate (J2) and methyl 5-chloro-4,5-didehydrojasmonate (J7) were synthesized in the laboratory of Professor J. H. Jung (College of Pharmacy, Pusan National University, Busan, Korea).⁶ Recombinant human IFN- γ , recombinant human TNF- α , fetal bovine serum (FBS), and RPMI1640 medium were obtained from GIBCO (Grand Island, NY, U.S.A.). The human

Fig. 4. Effects of J2 and J7 on MDC Production in IFN- γ - and TNF- α -Stimulated HaCaT Keratinocytes

HaCaT cells (2.0×10^5 cells/mL) were pre-incubated in unsupplemented medium for 18 h. MDC production was then determined in the supernatants of cells stimulated with IFN- γ (10 ng/mL) and TNF- α (10 ng/mL) in the presence or absence of analogues at the indicated concentrations for 24 h. MDC production was measured by ELISA, and cell viability was determined by the WST assay. The measurements of MDC were done in triplicate. Bars and error bars indicate the mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant differences compared with the positive control.

MDC enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems (St. Louis, MO, U.S.A.). Anti-phospho-ERK, anti-ERK, anti-phospho-JNK, anti-JNK, anti-p38, anti-I κ B- α , anti-phospho-NF- κ B p65, anti-NF- κ B p65, and anti-phospho-STAT1 antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-STAT1 and anti-phospho-p38 antibodies were purchased from Becton Dickinson (San Diego, CA, U.S.A.), and the anti- β -actin antibody was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade.

Cell Culture and Cell Viability Assays An immortalized human keratinocyte cell line, HaCaT, was cultured in RPMI1640 medium supplemented with 10% FBS and 100 U/mL penicillin-streptomycin in a humidified CO₂ incubator. Cell viability was determined by using the EZ-cytox enhanced cell viability assay kit (ITSBio, Inc., Seoul, Korea). Briefly, cells were seeded into the wells of a 96-well plate and treated with IFN- γ (10 ng/mL) and TNF- α (10 ng/mL) in the absence or presence of jasmonates or their synthetic analogues for 24 h. A solution of WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) was added to each well and incubated with the cells for 2 h in an incubator. After incubation, the absorbance of each well was measured at 450 nm with a VersaMax ELISA microplate reader (Molecular

Fig. 5. Effect of J7 on MDC mRNA Expression Level in HaCaT Keratinocytes

HaCaT cells (5.0×10^5 cells/mL) were pre-incubated for 18 h in unsupplemented culture medium. Cells were pre-treated with J7 at the indicated concentrations (2.5, 5, 10 μ M) for 2 h. Cells were then stimulated with IFN- γ (10 ng/mL) and TNF- α (10 ng/mL) in the presence of J7 for 6 h. The expression levels of MDC and GAPDH mRNA were determined by using RT-PCR.

Devices Inc., Sunnyvale, CA, U.S.A.).

ELISA Analysis Secretion of the MDC protein into the supernatant of cultured cells was measured by using an ELISA kit according to the manufacturer's instructions. Briefly, HaCaT cells were stimulated with IFN- γ and TNF- α in the presence of jasmonate derivatives and their analogues for 24 h. The cell culture medium was transferred to a 96-well culture plate coated with MDC antibody and treated according to the manufacturer's (R&D Systems) instructions. Absorbance at 450 nm was recorded by using the VersaMax ELISA microplate reader.

Extraction of Total RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was isolated from HaCaT cells by using the TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.) according to the manufacturer's instructions. Reverse transcription was performed with a First-Strand cDNA synthesis kit (Promega, Madison, WI, U.S.A.). Briefly, total RNA (1 μ g) was incubated with oligo(dT)₁₈ primer at 70°C for 5 min and cooled on ice for 5 min. After addition of the reverse transcription (RT) premix, reaction ingredients were incubated at 37°C for 60 min. Reactions were terminated by raising the temperature to 70°C for 15 min.

The PCR reaction was conducted by using i-Taq™ DNA polymerase (iNtRON Biotechnology, Kyungki-do, Korea) with the appropriate sense and antisense primers for MDC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences were as follows: MDC (F) 5'-GCA TGG CTC GCC TAC AGA CT-3', (R) 5'-GCA GGG AGG GAG GCA GAG GA-3'; GAPDH (F) 5'-GAG TCA ACG GAT TTG GTC GT-3', (R) 5'-GAC AAG CTT CCC GTT CTC AG-3'. PCR was performed with a C1000 instrument (Bio-Rad, Hercules, CA, U.S.A.). Thermal cycling conditions were set to denaturation at 94°C for 30 s, annealing at 55–60°C for 30 s, and extension at 72°C for 2 min, repeated 30 to 35 times, and a final incubation at 72°C for 10 min. The reaction products were visualized by electrophoresis on a 1.2% agarose gel (Promega) containing ethidium bromide, followed by ultraviolet

(A)

(B)

Fig. 6. Effect of J7 on STAT1 Phosphorylation in IFN- γ - and TNF- α -Stimulated HaCaT Keratinocytes

(A) HaCaT cells (5.0×10^5 cells/mL) were pre-treated with J7 (2.5, 5, 10 μ M) for 2 h. The phosphorylation (on Tyr701) of STAT1 was determined in cells stimulated with IFN- γ (10 ng/mL) and TNF- α (10 ng/mL) for 15 min. The phosphorylation or level of each protein in whole cell lysates was determined by Western blotting with the indicated antibodies. (B) HaCaT cells were pre-treated with J7 (10 μ M) for 2 h. The nuclear translocation of the STAT1 protein was determined in cells stimulated with IFN- γ and TNF- α for 60 min. Immunofluorescence staining for STAT1 was performed by using a primary antibody against STAT1, followed by a DyLight488-conjugated secondary antibody. The fluorescence was then identified by using a confocal microscope (FV500, Olympus Corp.), and the images were acquired at constant two-photon excitation microscopy (PMT), gain, offset, magnification (40 $\times$ oil immersion objective with a zoom factor of 1.5), and resolution. These data are representative of three independent experiments.

(UV) light illumination with the MiniLumi gel imaging system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis HaCaT cells were incubated with IFN- γ and TNF- α plus/minus jasmonate analogues, washed twice with ice-cold phosphate buffered saline (PBS), and then disrupted in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonident P-40, 2 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM NaVO₃, 10 mM NaF, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 25 μ g/mL leupeptin) on ice for 30 min. Cell lysates were centrifuged at 15000 rpm for 15 min at 4°C, and the supernatants were used for Western blotting. The total protein concentration of each sample was quantified via the Bio-Rad assay method (Bio-Rad). Extracts containing

Fig. 7. Effect of J7 on I κ B- α Degradation and NF- κ B Phosphorylation in IFN- γ - and TNF- α -Stimulated HaCaT Keratinocytes

(A) HaCaT cells (5.0×10^5 cells/mL) were pre-treated with J7 (2.5, 5, 10 μ M) for 2 h. The degradation of I κ B- α was determined in cells stimulated with IFN- γ (10 ng/mL) and TNF- α (10 ng/mL) for 15 min. (B) HaCaT cells were pre-treated with J7 (10 μ M) for 2 h. The translocation of NF- κ B was determined in cells stimulated with IFN- γ and TNF- α for 60 min. Immunofluorescence staining of NF- κ B was detected by using a primary antibody against NF- κ B, followed by a DyLight488-conjugated secondary antibody. The fluorescence was then visualized by using a confocal microscope (FV500, Olympus Corp.), and the images were acquired at constant PMT, gain, offset, magnification (40 $\times$ oil immersion objective with a zoom factor of 2.0), and resolution. These data are representative of three independent experiments. (C) HaCaT cells were pre-treated with J7 (2.5, 5, 10 μ M) for 2 h. The phosphorylation of ERK, JNK, and p38 MAPK was determined in cells stimulated with IFN- γ (10 ng/mL) and TNF- α (10 ng/mL) for 15 min. The phosphorylation or level of each protein in whole cell lysates was determined by Western blotting with the indicated antibodies.

30 μ g of protein were loaded next to a prestained protein-mass ladder (Bio-Rad) on a NuPAGE 4–12% bis-Tris gel (Invitrogen, Carlsbad, CA, U.S.A.). The proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane by using an iBlot gel transfer device (Invitrogen). The membrane was blocked with blocking buffer (5% skim milk in Tween 20-Tris buffered saline (TTBS)) for 1 h at room temperature, followed by overnight incubation at 4°C with the appropriate primary antibodies (anti-phospho-ERK, anti-ERK, anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-I κ B- α , anti-phospho-NF- κ B p65, anti-NF- κ B p65, anti-phospho-STAT1, anti-STAT1, and anti- β -actin antibodies). All antibodies were diluted in 1% bovine serum albumin (BSA) in TTBS buffer. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-primary antibody host immunoglobulin G (IgG) diluted 1:5000 for 1 h at room temperature. After washing again, immunoreactive bands were visualized with a western blot detection system (iNtRON Biotechnology) according to the manufacturer's instructions.

Confocal Microscopy Analysis HaCaT cells were seeded onto round 12mm coverslips in a 24-well plate, fixed with freshly prepared 3.7% paraformaldehyde for 30 min, and permeabilized with ice-cold 100% MeOH for 10 min at –20°C. After a 1 h incubation with 3% BSA/0.1% Triton X-100/PBS, the cells were incubated with primary anti-STAT-1 and anti-NF- κ B antibodies overnight at 4°C. The cells were washed and then incubated with DyLight488-conjugated donkey anti-rabbit (BioLegend, San Diego, CA, U.S.A.) secondary antibody for 60 min at room temperature. After several additional washing steps, the coverslips were mounted in VECTASHIELD mounting media with DAPI (Vector Labs, Burlingame, CA, U.S.A.). Fluorescently labeled STAT1 and NF- κ B were visualized by using a FV500 confocal microscope (Olympus Corp., Tokyo, Japan).

Statistical Analysis GelCapture Version 7.0.5 and ImageJ 1.45a software were used to transform images of Western blots and ethidium bromide stained gels into numerical values. Student's *t*-test and two-way analysis of variance (ANOVA) were used to determine the statistical significance of differences between experimental and control group values. All numerical data represent the mean $\pm$ standard deviation (S.D.).

Acknowledgment This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (MEST) (NRF-C1A-001–2012-0006306).

References

- 1) Fingrut O., Flescher E., *Leukemia*, **16**, 608–616 (2002).
- 2) Fingrut O., Reischer D., Rotem R., Goldin N., Altboum I., Zan-Bar I., Flescher E., *Br. J. Pharmacol.*, **146**, 800–808 (2005).
- 3) Flescher E., *Cancer Lett.*, **245**, 1–10 (2007).
- 4) Dang H. T., Lee H. J., Yoo E. S., Hong J., Bao B., Choi J. S., Jung J. H., *Bioorg. Med. Chem.*, **16**, 10228–10235 (2008).
- 5) Lee H. J., Maeng K., Dang H. T., Kang G. J., Ryou C., Jung J. H., Kang H. K., Prchal J. T., Yoo E. S., Yoon D., *J. Mol. Med. (Berl.)*, **89**, 83–90 (2011).
- 6) Dang H. T., Lee Y. M., Kang G. J., Yoo E. S., Hong J., Lee S. M., Lee S. K., Pyee Y., Chung H. J., Moon H. R., Kim H. S., Jung J. H., *Bioorg. Med. Chem.*, **20**, 4109–4116 (2012).
- 7) Boukamp P., Petrussevska R. T., Breitkreutz D., Hornung J., Markham A., Fusenig N. E., *J. Cell Biol.*, **106**, 761–771 (1988).
- 8) Horikawa T., Nakayama T., Hikita I., Yamada H., Fujisawa R., Bito T., Harada S., Fukunaga A., Chantry D., Gray P. W., Morita A., Suzuki R., Tezuka T., Ichihashi M., Yoshie O., *Int. Immunol.*, **14**, 767–773 (2002).
- 9) Cho K. A., Kim J. Y., Woo S. Y., Park H. J., Lee K. H., Pae C. U., *Ann. Dermatol.*, **24**, 398–405 (2012).
- 10) Kim Y., Lee S. K., Bae S., Kim H., Park Y., Chu N. K., Kim S. G., Kim H. R., Hwang Y. I., Kang J. S., Lee W. J., *Immunol. Lett.*, **149**, 110–118 (2012).
- 11) Kakinuma T., Nakamura K., Wakugawa M., Mitsui H., Tada Y., Saeki H., Torii H., Asahina A., Onai N., Matsushima K., Tamaki K., *J. Allergy Clin. Immunol.*, **107**, 535–541 (2001).
- 12) Kakinuma T., Nakamura K., Wakugawa M., Mitsui H., Tada Y., Saeki H., Torii H., Komine M., Asahina A., Tamaki K., *Clin. Exp. Immunol.*, **127**, 270–273 (2002).
- 13) Pivarsci A., Homey B., *Curr. Allergy Asthma Rep.*, **5**, 284–290 (2005).
- 14) Pease J. E., *Biochem. J.*, **434**, 11–24 (2011).
- 15) Imai T., Chantry D., Raport C. J., Wood C. L., Nishimura M., Godiska R., Yoshie O., Gray P. W., *J. Biol. Chem.*, **273**, 1764–1768 (1998).
- 16) Yamashita U., Kuroda E., *Crit. Rev. Immunol.*, **22**, 105–114 (2002).
- 17) Hijnen D., De Bruin-Weller M., Oosting B., Lebre C., De Jong E., Bruijnzeel-Koomen C., Knol E., *J. Allergy Clin. Immunol.*, **113**, 334–340 (2004).
- 18) Saeki H., Tamaki K., *J. Dermatol. Sci.*, **43**, 75–84 (2006).
- 19) Wakugawa M., Nakamura K., Kakinuma T., Tamaki K., *Drug News Perspect.*, **15**, 175–179 (2002).
- 20) Nakayama T., Hieshima K., Nagakubo D., Sato E., Nakayama M., Kawa K., Yoshie O., *J. Virol.*, **78**, 1665–1674 (2004).
- 21) Komine M., Kakinuma T., Kagami S., Hanakawa Y., Hashimoto K., Tamaki K., *J. Invest. Dermatol.*, **125**, 491–498 (2005).
- 22) Vestergaard C., Yoneyama H., Matsushima K., *Mol. Med. Today*, **6**, 209–210 (2000).
- 23) Najjar I., Fagard R., *Biochimie*, **92**, 425–444 (2010).
- 24) Akira S., *Stem Cells*, **17**, 138–146 (1999).
- 25) Kang G. J., Lee H. J., Yoon W. J., Yang E. J., Park S. S., Kang H. K., Park M. H., Yoo E. S., *Biomol. Ther.*, **16**, 394–402 (2008).
- 26) Jeong S. I., Choi B. M., Jang S. I., *Arch. Pharm. Res.*, **33**, 1867–1876 (2010).
- 27) Hongqin T., Xinyu L., Heng G., Lanfang X., Yongfang W., Shasha S., *Phytother. Res.*, **25**, 1678–1685 (2011).
- 28) Kang G. J., Han S. C., Yi E. J., Kang H. K., Yoo E. S., *Toxicol. Res.*, **27**, 173–180 (2011).
- 29) Ihle J. N., *Curr. Opin. Cell Biol.*, **13**, 211–217 (2001).
- 30) van Boxel-Dezaire A. H., Stark G. R., *Curr. Top. Microbiol. Immunol.*, **316**, 119–154 (2007).
- 31) Gough D. J., Levy D. E., Johnstone R. W., Clarke C. J., *Cytokine Growth Factor Rev.*, **19**, 383–394 (2008).
- 32) Demyanets S., Kaun C., Rychli K., Pfaffenberger S., Kastl S. P., Hoheninner P. J., Rega G., Katsaros K. M., Afonyushkin T., Bochkov V. N., Paireder M., Huk I., Maurer G., Huber K., Wojta J., *Basic Res. Cardiol.*, **106**, 217–231 (2011).
- 33) Kwon D. J., Bae Y. S., Ju S. M., Goh A. R., Youn G. S., Choi S. Y., Park J., *Biochem. Biophys. Res. Commun.*, **417**, 1254–1259 (2012).
- 34) Jeong S. I., Choi B. M., Jang S. I., *Arch. Pharm. Res.*, **33**, 1867–1876 (2010).