BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 23(3), 238-244 (2015)

Dieckol, a Component of *Ecklonia cava*, Suppresses the Production of MDC/CCL22 via Down-Regulating STAT1 Pathway in Interferon-γ Stimulated HaCaT Human Keratinocytes

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Abstract

Macrophage-derived chemokine, C-C motif chemokine 22 (MDC/CCL22), is one of the inflammatory chemokines that controls the movement of monocytes, monocyte-derived dendritic cells, and natural killer cells. Serum and skin MDC/CCL22 levels are elevated in atopic dermatitis, which suggests that the chemokines produced from keratinocytes are responsible for attracting inflammatory lymphocytes to the skin. A major signaling pathway in the interferon-γ (IFN-γ)-stimulated inflammation response involves the signal transducers and activators of transcription 1 (STAT1). In the present study, we investigated the anti-inflammatory effect of dieckol and its possible action mechanisms in the category of skin inflammation including atopic dermatitis. Dieckol inhibited MDC/CCL22 production induced by IFN-γ (10 ng/mL) in a dose dependent manner. Dieckol (5 and 10 μM) suppressed the phosphorylation and the nuclear translocation of STAT1. These results suggest that dieckol exhibits anti-inflammatory effect via the down-regulation of STAT1 activation.

Key Words: Dieckol, Inflammation, Keratinocyte, MDC/CCL22, STAT1

INTRODUCTION

Chemokine is a group of factors to control the activity of white blood cells, and serves to control the infiltration of inflammatory cells (Yoshie *et al*., 2001), being closely related to various pathological processes, such as inflammation, allergy, and infectious diseases. Chemokine is also known to be involved in generation and maturation of immune cells, and differentiation of T cells (Yoshie *et al*., 2001; Baumer *et al*., 2004). Macrophage-derived chemokine (MDC/CCL22) is a typical inflammatory chemokine and a ligand for CC chemokine receptor 4 (CCR4), which is predominantly expressed on Th2 lymphocytes, basophils and natural killer cells (Yamashita and Kuroda, 2002; Saeki and Tamaki, 2006). Previous studies have shown that the MDC level is elevated in the serum and skin lesions of patients with atopic dermatitis (AD), suggesting that keratinocyte-generated chemokines are key mediators in the drawing of inflammatory lymphocytes to the skin (Maeda *et al*., 2002; Shimada *et al*., 2004; Jahnz-Rozyk *et al*., 2005).

IFN-γ, one of the multifunctional cytokines that have antiviral, anti-tumor, and immunomodulatory effects is produced predominantly in T cells and NK cells (Farrar and Schreiber, 1993). Upon binding to IFN-γ, the IFN-γ receptor (IFN-γR1 and IFN-γR2) rapidly associates with the Janus tyrosine kinases JAK1 and JAK2. JAK1 and JAK2 phosphorylate one another and then subsequently phosphorylate the IFN-γ receptor, which leads to form a docking moiety for the cytoplasmic transcription factor named signal transducer and activator of transcription (STAT)1, a member of the STAT protein family (Ivashkiv and Hu, 2004; Best *et al*., 2005) . It has been found that STAT1 phosphorylation plays a critical role in IFN-mediated innate immunity to microbial infection, especially inflammatory responses (Ju *et al*., 2009). It is also known that IFN-γ also stimulates the activation of p38 and extracellular signal-regulated kinase 1/2 (ERK1/2), NF-κB (Pearson *et al*., 2001; Madonna *et al*., 2008).

Recently, a lot of effort has been made to discover biologically active components from marine plants. *Ecklonia cava* is

Open Access **http://dx.doi.org/10.4062/biomolther.2014.141**

Received Dec 15, 2014 **Revised** Jan 31, 2015 **Accepted** Feb 26, 2015
Published online May 1, 2015

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one of edible brown algae and is abundant along the coast of Jeju island. *E. cava* has been attended as a potential producer of diverse natural materials such as alginic acids, phlorotannins and fucoidans which show useful activities in bio-industrial areas including medicine, cosmetics, and functional foods (Wijesinghe *et al*., 2011; Kang *et al*., 2012b; Kim *et al*., 2014). Phlorotannins (eckol, dieckol, and bieckol) isolated from *E*. species are representative components of marine secondary metabolites. Among them, dieckol is a phlorotannin compound consisting of a dimeric structure of polyphenolic compound eckol (Park *et al*., 2013). Dieckol has shown anti-oxidant (Lee *et al*., 2012), anti-cancer (Park and Jeon. 2012), anti-diabetic (Lee *et al*., 2012; Kang *et al*., 2013), hepatoprotective (Kang *et al*., 2012a), and anti-inflammatory effects (Yayeh *et al*., 2014). There are several studies on the anti-inflammatory effects of dieckol in lipopolysaccharide (LPS)-induced murine macrophages (RAW264.7 cells) (Choi *et al*., 2014). However, the biological effects of dieckol and its action mechanisms in skin inflammation including atopic dermatitis are poorly understood.

In the present study, we aimed to explore the effect of dieckol on IFN-γ induced signaling pathways in HaCaT cells, as well as the link between specific pathways and inflammatory chemokine production.

MATERIALS AND METHODS

Reagents

Eckol and dieckol were provided by Professor Nam-Ho Lee (Jeju national university, Jeju, Korea) (Fig. 1). Human interferon- γ (hIFN-γ: recombinant *E.coli*) was purchased from Gibco (Grand Island, NY, USA), and MDC enzyme-linked immunosorbent assay (ELISA) duoset kit was obtained from R&D system (St. Louis, MO, USA). Anti-STAT1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-STAT1 antibody was purchased from Cell signaling (Beverly, MA, USA), and anti-β-actin antibody, Epigallocatechingallate (EGCG) and PD98059 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of reagent grade.

Cell culture and cell viability assay

Human adult low-calcium high-temperature (HaCaT) keratinocyte was obtained from the Amore Pacific Company (Gyeonggi-do, Republic of Korea). The cells were cultured in RPMI1640 medium supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. The cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2. Cell viability was determined using an EZ-cytox-enhanced cell viability assay kit (itsBIO, Korea) following the manufacturer's protocol. Briefly, cells were seeded into the wells of a 96-well plate and treated with IFN-γ (10 ng/mL) in the absence or presence of eckol or dieckol for 24 h. A solution (5 μL) of WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium) was added to each well and incubated for 1 h in an incubator. Then, the absorbance of each well was measured at 450 nm with a VersaMax ELISA microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

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 in the presence of dieckol or other test samples 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.

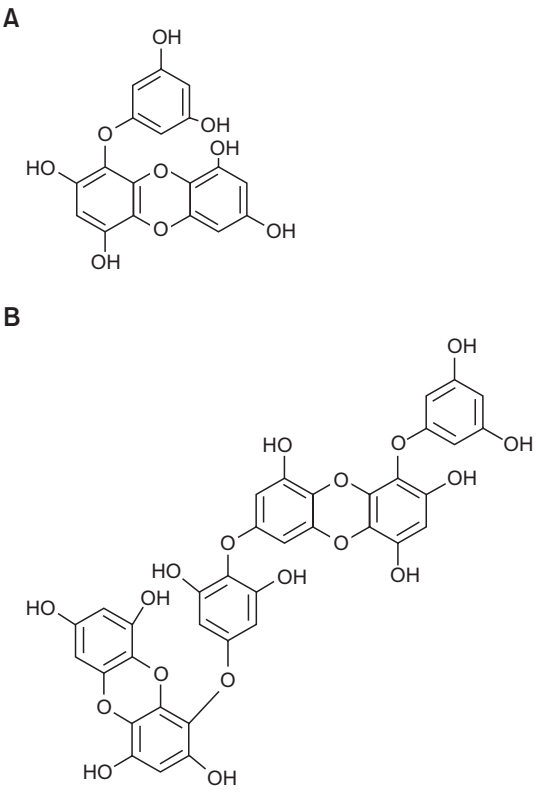

Fig. 1. Chemical structures of eckol and dieckol. (A) Eckol (B) Dieckol.

Western blot analysis

HaCaT cells were 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 μg/mL leupeptin) on ice for 30 min. Cell lysates were centrifuged at 15,000 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 30 μg of protein were loaded next to a prestained protein-mass ladder (Bio-Rad) on a NuPAGE 4-12% bis-Tris gel (Invitrogen, Carlsbad, CA, USA). 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-STAT1, anti-STAT1, anti-phospho-ERK, anti-ERK, anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38,

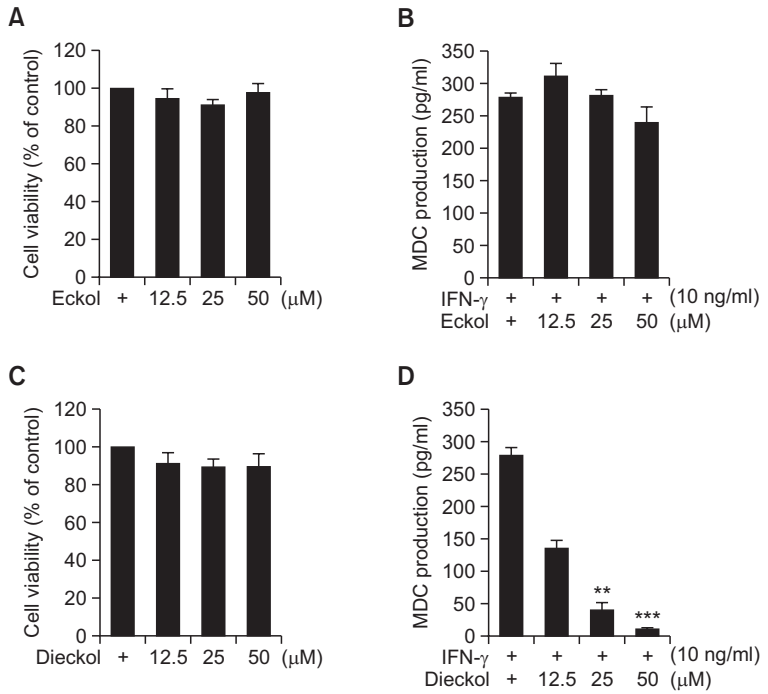

Fig. 2. Effects of eckol and dieckol on the cell viability and MDC production in HaCaT human keratinocytes. (A) and (C) HaCaT cells were treated with indicated concentrations of eckol or dieckol (12.5, 25, and 50 μM) for 24 h, and cell viability was assayed by WST assay. (B) and (D) HaCaT cells (2.0×10^5 cells/mL) were stimulated with IFN-γ (10 ng/mL) in the presence or absence of eckol or dieckol for 24 h. The amounts of MDC were measured from the culture supernatants by ELISA. Data are mean ± S.D. of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$ vs. sample-untreated cells in the presence of IFN-γ.

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 coverslips in a 6-well plate and incubated were stimulated with IFN-γ in the presence of dieckol for 24 h. 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-STAT1 antibodies overnight at 4°C. The cells were washed and then incubated with DyLight488-conjugated donkey anti-rabbit (BioLegend, San Diego, CA, USA) secondary antibody for 30 min at room temperature. After several additional washing steps, the coverslips were mounted in VECTASHIELD mounting media with DAPI (Vector Labs, Burlingame, CA, USA). Fluorescently labeled STAT1 was visualized by using a FV500 confocal microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis

ImageJ 1.47n software was used to transform images of Western blots into numerical values. Student's *t*-test was determined the statistical significance of differences. All numerical data represent the mean ± standard deviation (S.D.).

RESULTS

Effects of eckol and dieckol on MDC production in IFN-γ-stimulated HaCaT keratinocytes

We first examined the cell cytotoxicity of eckol and dieckol against HaCaT cells. The cells were treated with different concentrations of eckol and dieckol (12.5, 25, and 50 μM) for 24 h. Cell viability was determined using an EZ-cytox-enhanced cell viability assay kit. As shown in Fig. 2A and 2C, eckol and dieckol did not exhibit cytotoxicity to HaCaT keratinocytes at the tested concentrations. Then, we evaluated the inhibitory effects of eckol and dieckol on inflammatory chemokine (MDC) production in IFN-γ (10 ng/mL)- stimulated HaCaT keratinocytes. The basal level of MDC production is 118.0 ± 8.3 pg/mL, and 278.6 ± 6.0 pg/mL of MDC was produced by IFN-γ treatment (10 ng/mL). Interestingly, eckol failed to inhibit the cytokine-stimulated production of MDC (Fig. 2B). However, dieckol significantly suppressed the production of MDC by IFN-γ in a concentration-dependent manner. Especially, 12.5 uM dieckol strongly suppressed by 135 ± 12.7 pg/mL (almost the basal level) of MDC production in IFN-γ-stimulated HaCaT keratinocyte (Fig. 2D).

Effect of dieckol on STAT1 activation in IFN-γ-stimulated HaCaT keratinocytes

STAT1 protein is a crucial and specific regulator of IFN-γ-induced signals that controls the transcription of target genes, including MDC (Ivashkiv and Hu. 2004; Best *et al*., 2005; Kang *et al*., 2008; Rauch *et al*., 2013). Therefore, we tested the effect of dieckol on the activation of STAT1 in IFN-γ- treated HaCaT keratinocytes and detected a high level of phosphorylated

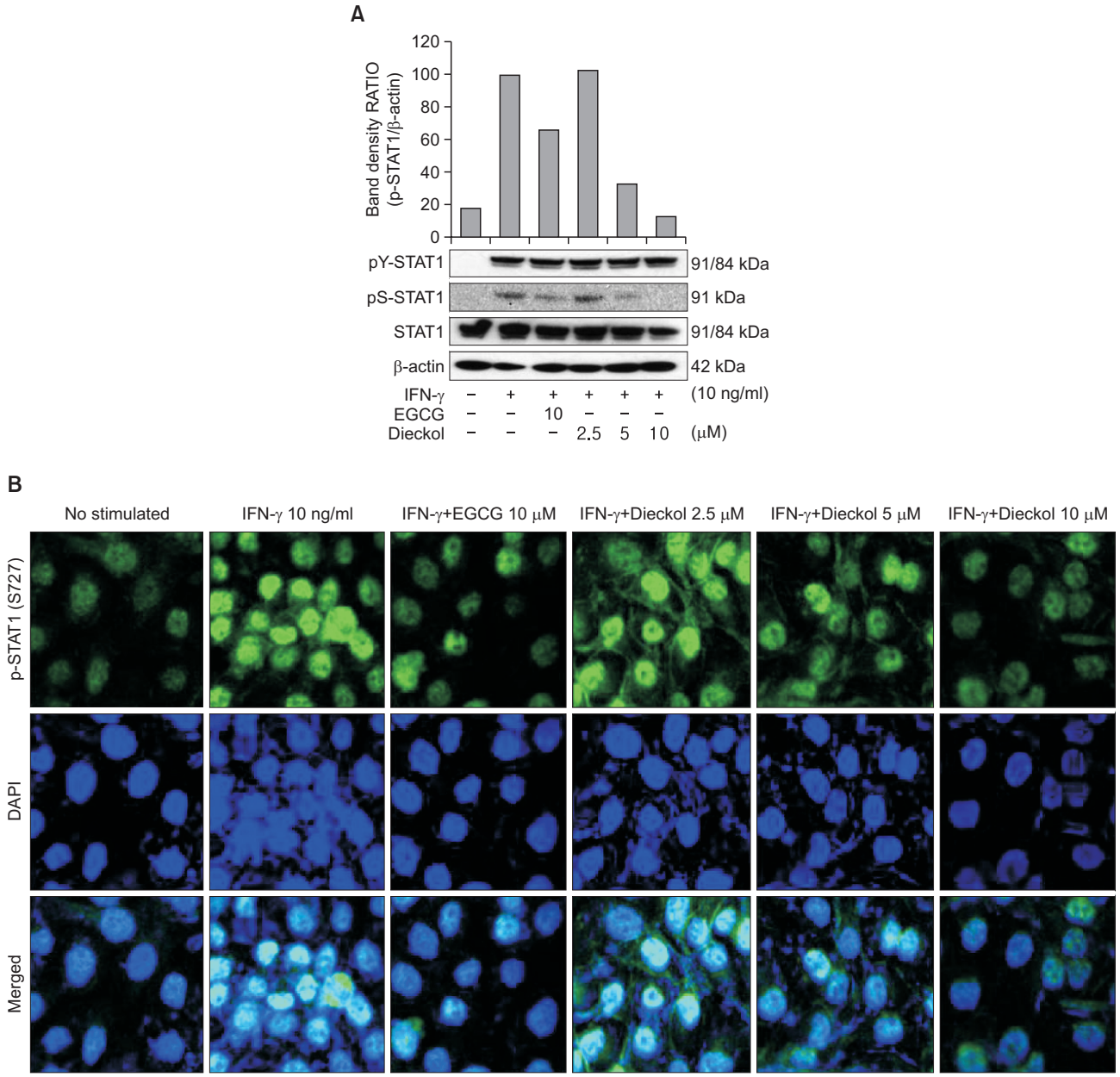

Fig. 3. Effect of dieckol on the phosphorylation and nuclear translocation of STAT1 in IFN-γ -stimulated HaCaT human keratinocytes. (A) HaCaT cells (5.0×10^5 cells/mL) were pretreated with epigallocatechingallate (EGCG; 10 μM) or dieckol (2.5, 5, 10 μM) for 30 min. The phosphorylation of STAT1 was determined in cells stimulated with IFN-γ (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 EGCG (10 μM) or dieckol (2.5, 5, 10 μM) for 2 h. The nuclear translocation of the STAT1 protein was determined in cells stimulated with IFN-γ for 20 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× oil immersion objective with a zoom factor of 1.5), and resolution.

STAT1 at 15 min after cytokine treatment. Pre-treatment of the cells with dieckol for 30 min dose-dependently suppressed STAT1 (serine 727) phosphorylation (Fig. 3A). In addition, microscopy results showed that IFN-γ led to nuclear translocation of STAT1 within 1 h, and dieckol (5, 10 μM) suppressed the nuclear translocation of phosphorylated STAT1 (serine 727) (Fig. 3B). These results suggest that the inhibitory effect of dieckol on the production of MDC occurs through inhibiting the activation and nuclear translocation of STAT1.

Effect of dieckol on MAP kinases pathway in IFN-γ-stimulated HaCaT keratinocytes

The MAPKs pathway is reportedly involved in the production of inflammatory chemokines, IFN-γ activates receptor-

associated MAPKs depending on the cell type (Pearson *et al*., 2001; Madonna *et al*., 2008). We thus investigated the involvement of these signaling kinases in IFN-γ induced MDC production in HaCaT cells. We first determined time dependent activation of three MAPKs (ERK, JNK, p38) after IFN-γ treatment. As illustrated in Fig. 4A. IFN-γ induced the phosphorylation of ERK at 5 min, while there was no effect on the phosphorylation of JNK and p38. Then we examined the inhibitory effect of dieckol on the ERK activation in IFN-γ-stimulated HaCaT cells. PD98059 (a specific ERK inhibitor, 10 μM), strongly suppressed ERK phosphorylation, but, dieckol did not inhibit ERK phosphorylation (Fig. 4B). To confirm whether MAPKs pathway associate with MDC production, we determined the MDC level after the treatment of known MAPKs inhibitors in IFN-γ-stimulated HaCaT cells. SP600125 (a specific JNK inhibitor) and PD98059 (a specific ERK inhibitor), and SB203580 (a specific p38 inhibitor) did not affect on the MDC production induced by IFN-γ (Fig. 4C). These results suggest that MAPKs pathway does not contribute on the production of MDC in IFN-γ stimulated HaCaT cells.

DISCUSSION

Various marine bio-resources have recently been explored because of the investigations of active components in pharmaceutical and functional food areas (Kim and Himaya. 2011; Jung *et al*., 2013). Dieckol was discovered during the determination of anti-inflammatory materials from marine plants living in the Jeju coastal area. Dieckol, one of various bioactive phlorotannins of Ecklonia cava (e.g., eckol, 6, 6-bieckol, dieckol, and phloroglucinol), is suggested to elicit anti-diabetic, antioxidant, anti-tumorigenic, and anti-inflammatory activities (Lee *et al*., 2012; Park and Jeon. 2012; Kang *et al*., 2013; Yayeh *et al*., 2014; Choi *et al*., 2014).

In the present study, we investigated the anti-inflammatory effect of dieckol and its possible action mechanisms in the category of skin inflammation including atopic dermatitis. First we examined the effect of the two phlorotannin compounds (eckol and dieckol) on the MDC production in IFN-γ-stimulated HaCaT keratinocytes. Dieckol suppressed MDC production in a dose dependent manner, while eckol was not effective.

The promoter region of the MDC gene contains binding units for signal transducers and activators of transcription (STAT), activating protein (AP)-1, and NF-κB, and these factors may mediate the transcription of the MDC (Pearson *et al*., 2001; Ivashkiv and Hu. 2004; Best *et al*., 2005; Madonna *et al*., 2008; Qi *et al*., 2009). IFN-γ acts via two IFN receptors subunits (IFNGR1 and IFNGR2), which form a heterotetramer on the cell surface, and activates various signaling cascades. Phosphorylated STAT1 functions as a transcription factor that activates the primary genes related to inflammatory responses. Therefore, the STAT1 protein is a crucial and specific regulator of IFN-γ signaling that controls the transcription of target genes, including MDC (Ivashkiv and Hu. 2004; Best *et al*., 2005). It has been found that STAT1 phosphorylation plays a critical role in IFN-mediated innate immunity to microbial infection (van den Broek *et al*., 1995; Han *et al*., 2002; Semper *et al*., 2014). After phosphorylation, STAT1 homodimerizes and translocates to the nucleus, and promotes gene transcription via binding to IFN-γ-activated genes. STAT1 homodimerization is preferentially mediated by the binding of the phosphory-

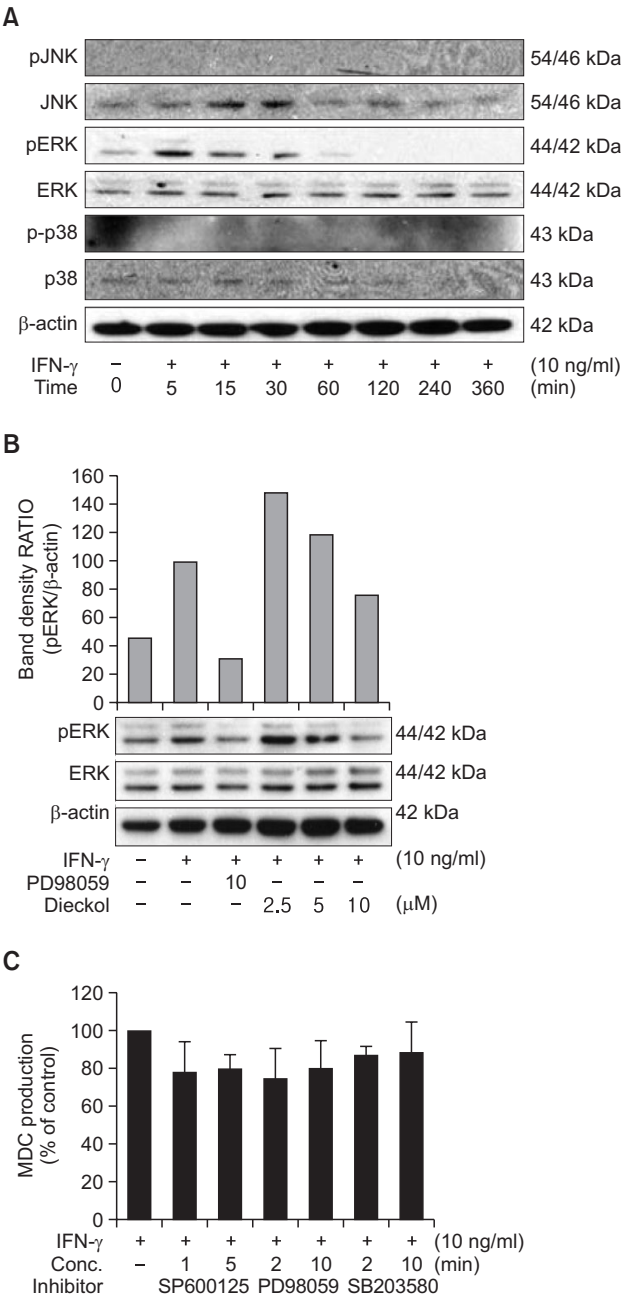

Fig. 4. Effect of dieckol on the MAPKs pathway in IFN-γ -stimulated HaCaT human keratinocytes. (A) HaCaT cells (5.0×10^5 cells/mL) were stimulated with IFN-γ (10 ng/mL) for indicated times (0-360 mins). Whole cell lysates were obtained at indicated time points. The phosphorylations of p38, JNK, and ERK were assessed by Western blotting from whole cell lysates. (B) HaCaT cells (5.0×10^5 cells/mL) were pre-treated with PD98059 (a specific ERK inhibitor, 10 μM) or dieckol (2.5, 5, 10 μM) for 2 h and stimulated by IFN-γ (10 ng/mL) for 5 mins. The phosphorylation of ERK was determined by Western blotting of whole cell lysates. (C) HaCaT cells (2.0×10^5 cells/mL) were stimulated with IFN-γ (10 ng/mL) in the presence or absence of the specific MAPK inhibitors (SP600125; JNK inhibitor, PD98059; ERK inhibitor, SB203580; p38 inhibitor) for 24 h. The amounts of MDC were measured from the culture supernatants by ELISA. Data are mean ± S.D. of three independent experiments.

lated STAT1 (at tyrosine 701) to the Src homology 2 domain of another. However, the binding of the phosphorylated serine 727 of STAT1 is required for maximal transcriptional activity (Decker and Kovarik. 2000; Ivashkiv and Hu. 2004; Best *et al*., 2005). In our study, dieckol suppressed STAT1 (at serine 727) phosphorylation induced by IFN-γ in a dose dependent manner. The effect of dieckol on the STAT1 phosphorylation is stronger than that of EGCG, a specific STAT inhibitor, at the same concentration (10 μM). In addition, microscopy results showed that dieckol (5, 10 μM) suppressed the nuclear translocation of phosphorylated STAT1 (at serine 727).

The MAPK pathway is reportedly involved in the production of inflammatory chemokines. Several plant extracts and compounds have been shown to inhibit the actions of inflammatory chemokines via the regulation of mitogen-activated protein kinase (MAPK) cascades. (Pearson *et al*., 2001; Madonna *et al*., 2008; Chae *et al*., 2013; Yoon *et al*., 2013). To determine whether the MAPKs (ERK, JNK, p38) signaling is involved in IFN-γ induced MDC production in HaCaT cells, we first examined time dependent activation of three MAPKs (ERK, JNK, p38) after IFN-γ treatment. IFN-γ induced the phosphorylation of ERK at 5 min, while did not affect on the phosphorylation of JNK and P-38. Then, we focused on the inhibitory effect of dieckol on the ERK activation in IFN-γ-stimulated HaCaT cells, but, the effect of dieckol was not significant. To confirm whether MAPKs pathway associate with MDC production, we determined the MDC levels after the treatment of known MAPKs inhibitors in IFN-γ-stimulated HaCaT cells. SP600125 (a specific JNK inhibitor) and PD98059 (a specific ERK inhibitor), and SB203580 (a specific p38 inhibitor) did not affect on the MDC production induced by IFN-γ. These results suggest that MAPKs pathway does not contribute significantly on the production of MDC in IFN-γ stimulated HaCaT cells.

Taken together, of the two phlorotannin compounds (eckol and dieckol) tested in this study, only dieckol strongly suppressed MDC production in HaCaT human keratinocytes. Dieckol also inhibited the activation of STAT1, a major transcription factor that activates the primary genes induced by IFN-γ. In addition, MAPKs pathway did not affect on the production of MDC in IFN-γ stimulated HaCaT cells. These results provide new evidence regarding anti-inflammatory potential of dieckol and its action mechanism in a skin cell line.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (MEST) (NRF-C1ABA001-2011-0021039).

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