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Anti-Inflammatory Effect of Quercetagetin, an Active Component of Immature *Citrus unshiu*, in HaCaT Human Keratinocytes

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Abstract

Citrus fruit contain various flavonoids that have multiple biological activities. However, the content of these flavonoids are changed during maturation and immature *Citrus* is known to contain larger amounts than mature. Chemokines are significant mediators for cell migration, while thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) are well known as the typical inflammatory chemokines in atopic dermatitis (AD), a pruritic and chronic inflammatory skin disease. We reported recently that the EtOH extract of immature *Citrus unshiu* inhibits TARC and MDC production. Therefore, we investigated the activity of flavonoids contained in immature *Citrus* on TARC and MDC levels. As a result, among the various flavonoids, quercetagetin has stronger inhibitory effects on the protein and mRNA expression of TARC and MDC than other flavonoids. Quercetagetin particularly has better activity on TARC and MDC level than quercetin. In HPLC analysis, the standard peak of quercetagetin matches the peaks of extract of immature *C. unshiu*. This suggests that quercetagetin is an anti-inflammatory component in immature *C. unshiu*.

Key Words: Quercetagetin, Flavonoids, Immature *Citrus unshiu*, TARC (CCL17), MDC (CCL22)

INTRODUCTION

Quercetagetin is a characteristic flavonol compound that has an additional 6-OH group based on the molecular structure of the flavone backbone (2-phenyl-1,4-benzopyrone). It has many effects, including antifungal, antibacterial, and antioxidant (Schmeda-Hirschmann *et al.,* 2004; Céspedes *et al.,* 2006). Furthermore, quercetagetin isolated from *Citrus unshiu* peel as citrus by-product showed a strong protective effect on DNA damage induced by H_2O_2 (Yang *et al.,* 2011). There is a recent report that quercetagetin inhibits the UVB-induced phosphorylation of c-Jun and AKT by binding to the JNK1 and PI3-K (Baek *et al.,* 2013).

Several studies highlighted *Citrus* genus as important health-promoting fruits rich in phenolic compounds as well as vitamins, minerals, dietary fiber, essential oils, carotenoids, and limonoids (Nogata *et al.,* 2006). Flavonoids are a class of secondary metabolites and impart significant protective biological effects including anti-cancer, anti-viral, anti-inflammatory and anti-atherogenic activities (Middleton *et al.,* 2000; Yang *et al.,* 2011). Interestingly, flavonoid content changes during the maturation of *Citrus* and bioactive flavonoids, like hesperidin, neohesperidin, narirutin, quercetagetin, were highly concentrated in immature *Citrus* (Kim *et al.,* 2009). Based on these reports, we recently reported that the EtOH extract of immature *C. unshiu* inhibits the various symptoms of an AD animal model (Kang *et al.,* 2011).

Chemokines are small proteins (67 to 127 amino acids) released from various cell types that regulate the traffic of immune cells to inflammatory or infectious site. Various inflammatory cytokines stimulate the expression of chemokines and specific inflammatory chemokines are found in the serum of atopic dermatitis (AD) patients (Kakinuma *et al.,* 2001; Pivarcsi and Homey, 2005). Among them, thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) are typical inflammatory chemokines and ligands 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 showed that levels of TARC and MDC in serum and skin lesions are elevated in AD, suggesting that chemokines produced from keratinocytes could

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be the key molecule in attracting inflammatory lymphocytes to the skin (Leung *et al.,* 2003; Hijnen *et al.,* 2004; Pease, 2011).

Therefore, we investigated the effect of various flavonoids, especially quercetagetin, on the production of TARC and MDC in IFN-γ and TNF-α-stimulated HaCaT human keratinocytes and we forecast the action mechanism of quercetagetin that is thought to be an active compound in immature *Citrus unshiu*.

MATERIALS AND METHODS

Chemicals and reagents

Hesperidin, hesperetin, neohesperidin, naringenin, naringin, nobiletin, tangeretin, and quercitrin were obtained from Sigma (St. Louis, MO, USA). Quercetagetin and quercetin were obtained from Extrasynthese (France) and Cayman Chemical (Ann Arbor, Mi, USA), respectively. Recombinant human IFN-γ and TNF-α, fetal bovine serum (FBS), and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). Human TARC and MDC enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (St. Louis, MO, USA). TARC and MDC primers for end point PCR were obtained from Bioneer (Korea). β-actin primers were purchased from Bionex (Korea). Jak inhibitor I (Jak I) was obtained from Calbiochem (La Jolla, CA, USA). Anti-phospho-STAT1 (Tyr701 and Ser727) was purchased from Cell Signaling (Beverly, MA, USA); anti-STAT1 from Becton Dickinson (San Diego, CA, USA); and β-actin from Sigma. All other chemicals were of reagent grade.

Cell culture and cell viability assays

An immortalized human keratinocyte cell line, HaCaT, was cultured in RPMI1640 supplemented with 10% FBS and 100 U/ml penicillin-streptomycin in a humidified CO_2 incubator. Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assays. HaCaT cells were stimulated with IFN-γ and TNF-α in the absence or presence of immature *C. unshiu* extract and various flavonoids. After incubating for 24 hrs, cells were treated with 10 μl MTT for 4 hrs. The formazan precipitate was dissolved in 200 μl of dimethyl sulfoxide (DMSO) for 30 min, and the absorbance of the contents of each well was measured at 540 nm using a microplate reader.

ELISA

Production of TARC and MDC proteins in the supernatant of cultured cells was measured using ELISA kits according to the manufacturer's instructions. HaCaT cells were stimulated by IFN-γ and TNF-α in the presence of various flavonoids for 24 hr. The cell culture medium was transferred to TARC or MDC antibody-coated 96-well culture plate and treated according to manufacturer's instruction. Absorbance at 450 nm was recorded using VersaMax ELISA microplate reader (Molecular Devices, CA, USA).

Extraction of total RNA and RT-PCR

Total RNA was isolated using the TRI reagent (Molecular Research Center, INC., Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription was performed using a First-Strand cDNA synthesis kit (Promega, Madison, WI, USA). Total RNA (1 μg) 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 using i-Taq™ DNA polymerase (iNtRON Biotechnology, Korea) with the appropriate sense and antisense primers for TARC, MDC, and β-actin. The primer sequences were as follows: TARC primer sequence (F) 5'-ATG GCC CCA CTG AAG ATG CT-3', (R) 5'-TGA ACA CCA ACG GTG GAG GT-3' (351 bp); MDC primer sequence (F) 5'-GCA TGG CTC GCC TAC AGA CT-3', (R) 5'-GCA GGG AGG GAG GCA GAG GA-3' (497 bp); β-actin primer sequence (F) 5'-ATG GGT CAG AAG GAT TCC TAT G-3', (R) 5'-CAG CTC GTA GCT CTT CTC CA-3' (588 bp). PCR was performed using a C1000 instrument (Bio-Rad, Hercules, CA, USA). Thermal cycling conditions were set to 94°C for 30 sec, annealing at 55-60°C for 30 sec, and extending at 72°C for 2 min, repeated 30 to 35 times, and followed by incubation at 72°C for 10 min. The reaction products were visualized by electrophoresis on a 1.2% agarose gel (Promega) and UV light illumination after staining with ethidium bromide. The relative intensity was analyzed using Quantity One software, version 4.2.1 (Bio-Rad).

Real-time quantitative PCR was performed with a TaqMan® Universal Master Mix II (Applied Biosystems, Piscataway, NJ) with a StepOnePlus™ Real-Time PCR (Applied Biosystems). Real-time PCR for the relative quantification of target gene copy numbers in relation to β-actin expression was conducted using the following primers and probes: 5'-GTA CCA GAC ATC TGA GG-3' (forward), 5'-ATT CTT CAC TCT CTT GTT GT-3' (reverse), and 5'-Fam-TCC AGG GAT GCC ATC GTK TTT-BHQ-1-3' (Taqman probe) for TARC; 5'-TGG ATC GCC TAC AGA CT-3' (forward), 5'-GTA ATC ACG GCA GCA GA-3' (reverse), and 5'-Fam-CTC GTC CTY CTT GCT GTG GCR-BHQ-1-3' (Taqman probe) for MDC; 5'-CCA ACC GTG AAA AGA TG-3' (forward), 5'-CGG AGT CCA TCA CAA TG-3' (reverse), and 5'-Fam-ACC TTC AAC ACC CCA GCC A-BHQ-1-3' (Taqman probe) for β-actin. Real-time PCR results were expressed using the StepOne™ software (Applied Biosystems) that measures amplification of the target and the endogenous control in experimental samples and in a reference sample. Measurements were normalized using the endogenous control.

SDS-PAGE and western blot analysis

HaCaT cells were washed twice with ice-cold PBS and then disrupted in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonident P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin] on ice for 30 min. Cell lysates were centrifuged at 15,000 rpm for 15 min at 4°C and supernatants were used for western blotting. The total protein concentration of each sample was quantified by 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 using an iBlot gel transfer device (Invitrogen). The membrane was blocked with blocking buffer (5% skim milk in TTBS) for 1 hr at room temperature (RT), followed by incubation with primary antibodies (1:1,000) overnight at 4°C. All antibodies were diluted in 1% BSA in TTBS buffer. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated

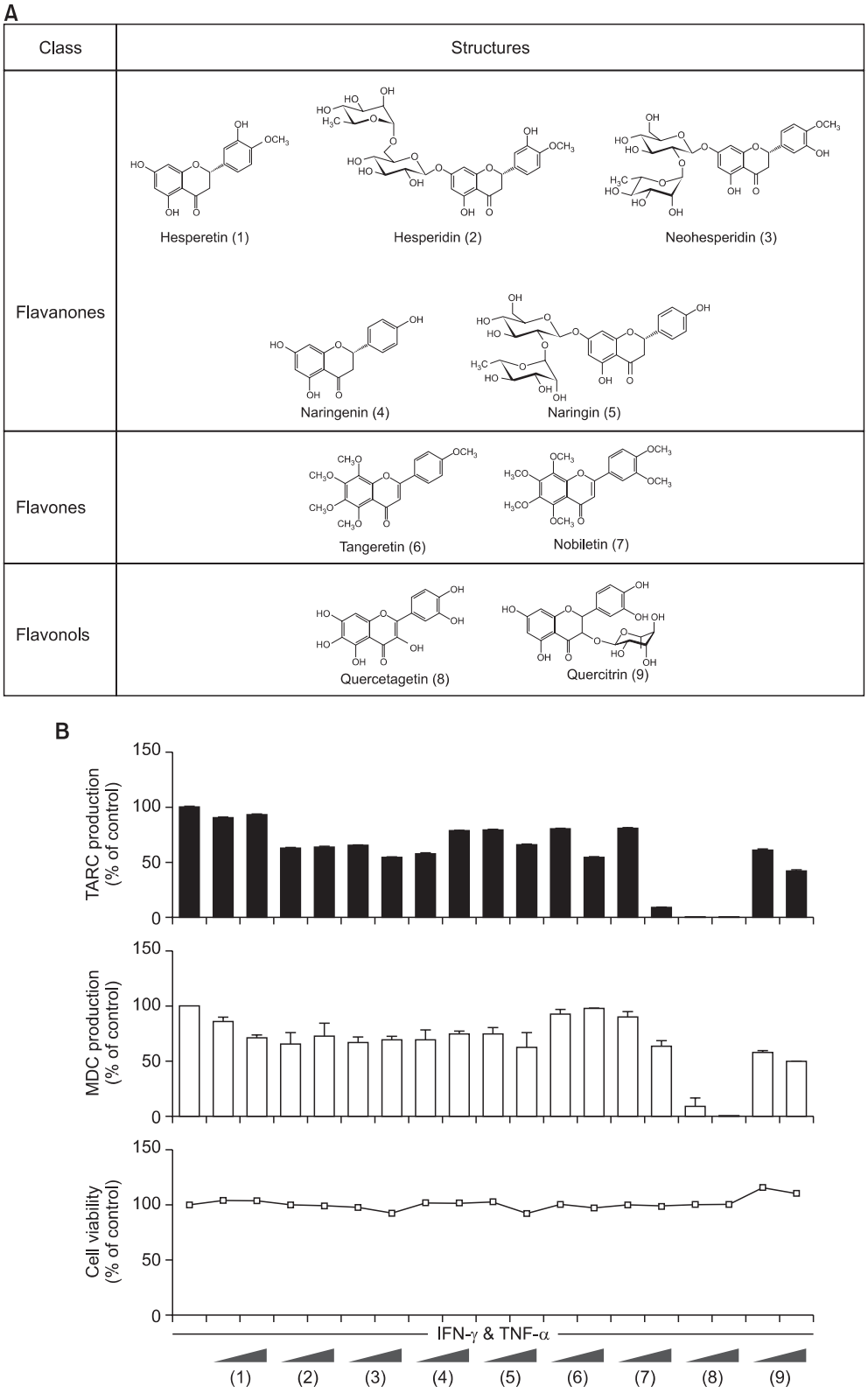

Fig. 1. Effects of various flavonoids contained within immature *Citrus unshiu* on inflammatory chemokines production and cell viability in IFN-γ- and TNF-α-stimulated HaCaT human keratinocytes. (A) Chemical structures of flavonoids. (B) HaCaT cells (3.0×10^5 cells/ml) were pre-incubated with unsupplemented culture medium for 18 hr. They were then stimulated with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) for 24 hr in the presence of flavonoids (25, 50 μM). TARC and MDC productions were determined from the culture supernatants by ELISA assay, and cell viability was analyzed by MTT assay. The measurements were made in triplicate. Error bars indicate mean ± S.D.

anti-primary Ab host IgG diluted 1:5,000 for 1 hr at RT. After washing again, the result was visualized with a western blot detection system (iNtRON Biotechnology, Korea) according to the manufacturer's instructions.

HPLC analysis

HPLC analysis was done to identify the existence of quercetagetin and quercetin in the EtOH extract of immature *C. unshiu*, used in our previous study (Kang *et al.,* 2011). This assay was conducted by Jeju Technopark (http://bio.jejutp.or.kr/english/, Korea).

Statistical analysis

Quantity One version 4.2.1 and Image-Pro plus version 4.5 software were used to transform images into numerical values. Student's *t*-test and two-way analysis of variance were used to determine the statistical significance of differences between experimental and control group values. Data represent the mean ± standard deviation. Null hypotheses of no difference were rejected if *p*-values were less than .05.

RESULTS

Effects of various flavonoids abundantly contained in immature *C. unshiu* on TARC and MDC production in HaCaT human keratinocytes

We reported recently that the EtOH extract of immature *Citrus unshiu* inhibits IFN-γ and TNF-α-induced inflammatory chemokines, TARC and MDC, in HaCaT keratinocytes. To identify the active compounds in immature *Citrus unshiu*, we investigated the inhibitory effects of various flavonoids found in immature *Citrus* on the production of TARC and MDC in HaCaT human keratinocytes.

First, we compared flavanones known to be contained in immature Citrus, like hesperetin (1), hesperidin (2), neohesperidin (3), naringenin (4), and naringin (5). Not all flavanones affected cell viability. However, neohesperidin and naringin showed a weak inhibitory effect on cell viability at 50 μM (Fig. 1B, bottom). IFN-γ and TNF-α stimulated HaCaT keratinocytes were treated with several flavonoids – hesperetin, hesperidin, neohesperidin, naringenin or naringin – for 24 hr. Then TARC and MDC production in the supernatant was measured by

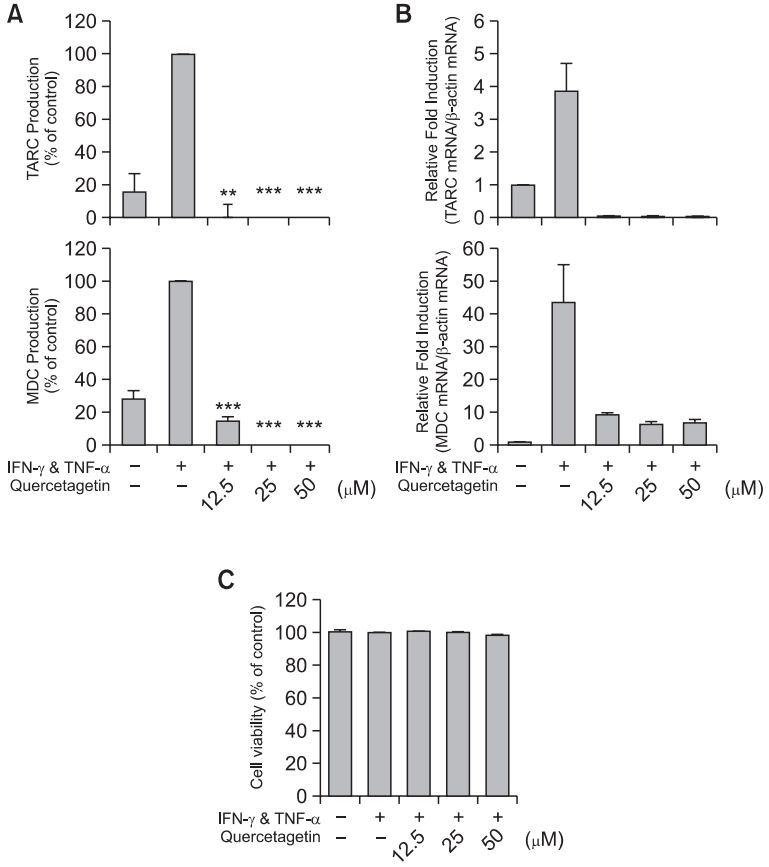

Fig. 2. Effect of quercetagetin on TARC and MDC expressions in HaCaT human keratinocytes. HaCaT cells (5.0×10^5 cells/ml) were pre-incubated for 18 hr in unsupplemented culture medium. (A) TARC and MDC productions were measured in the culture supernatant of the cells stimulated with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) for 24 hr in the presence of quercetagetin (12.5, 25, 50 μM) by an ELISA method. The measurements of TARC and MDC were done in triplicate. Error bars indicate ± S.D. ***p*<0.01, ****p*<0.001. (B) Cells were pre-treated with quercetagetin at the indicated concentrations for 2 hr (12.5, 25, 50 μM). Cells were then stimulated with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) in the presence of quercetagetin for 18 hr. The expression levels of TARC, MDC, and β-actin mRNA were examined by real-time RT-PCR in triplicate. (C) HaCaT cells (3.0×10^5 cells/ml) were pre-incubated with unsupplemented culture medium for 18 hr and then they were stimulated with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) for 24 hr in the presence of quercetagetin (12.5, 25, 50 μM). Cell viability was analyzed by the MTT assay. The measurements were made in triplicate. Error bars indicate mean ± S.D.

ELISA. Hesperetin weakly inhibited MDC production, but not TARC production, and hesperidin, neohesperidin, naringenin, and naringin weakly inhibited TARC and MDC production in HaCaT cells (Fig. 1B, top and middle).

We then examined the effects of flavones, tangeretin (6) and nobiletin (7), on the production of TARC and MDC in HaCaT human keratinocytes. Tangeretin and nobiletin also had no effect on cell viability at 25 and 50 μM (Fig. 1B, bottom). As a result, tangeretin showed only a weak inhibitory effect on TARC production. However, nobiletin at 50 μM strongly inhibited TARC production and weakly diminished on MDC production (Fig. 1B, top and middle).

We lastly investigated the effects of flavonols, quercetagetin (8), and quercitrin (9), on the production of TARC and MDC in HaCaT human keratinocytes. In a cell viability assay, quercetagetin, and quercitrin had no effect (Fig. 1B, bottom). Treatment with quercetagetin strongly inhibited the induction of TARC and MDC, but quercitrin weakly decreased TARC and MDC production (Fig. 1B, top and middle). From these results, we expected that quecetagetin might be an active compound in immature *Citrus unshiu*.

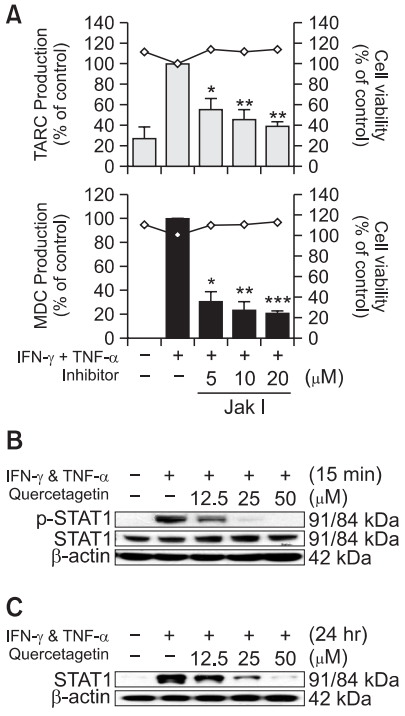

Fig. 3. Effect of quercetagetin on STAT1 signal related with TARC and MDC expressions in IFN-γ- and TNF-α-stimulated HaCaT human keratinocytes. (A) HaCaT cells (5.0×10^5 cells/ml) were pre-incubated for 18 hr in unsupplemented culture medium. The cells were then stimulated with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) in the presence of Jak I (Jak1/2 inhibitor) with indicated concentrations for 24 hr. The measurements of TARC and MDC were determined from culture supernatant by the ELISA in triplicate. Error bars indicate ± S.D. **p*<0.05; ***p*<0.01; ****p*<0.001 (B) HaCaT cells (5.0×10^5 cells/ml) were pre-treated with quercetagetin (50 μM) for 2 hr. The phosphorylation (on Tyr701) of STAT1 was determined in cells stimulated by IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) in the presence of quercetagetin (12.5, 25, 50 μM) for 15 min. (C) The level of STAT1 was determined from cells stimulated by IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) for 24 hr. The phosphorylation or level of each protein in whole-cell lysates were determined by western blotting with the indicated antibodies.

Effect of quercetagetin on the protein productions and the mRNA expressions of TARC and MDC in HaCaT human keratinocytes

Subsequently, we scrutinized whether quercetagetin inhibits its TARC and MDC expression in IFN-γ and TNF-α stimulated HaCaT human keratinocytes. Treatment with quercetagetin dose-dependently inhibited the induction of TARC and MDC protein (Fig. 2A) in supernatants from cultured HaCaT cells that had been stimulated with IFN-γ and TNF-α for 24 hrs. We confirmed the effect of quercetagetin (12.5, 25, 50 μM) on TARC and MDC mRNA expression by real-time RT-PCR. TARC or MDC mRNA level increased about 4- or 40-fold by IFN-γ and TNF-α stimulation for 18 hr. However, quercetagetin did inhibit mRNA levels of TARC and MDC by up to 80-90% (Fig. 2B). In the test by end-point RT-PCR, quercetagetin clearly suppressed the mRNA expression of TARC and MDC in a dose-dependent manner (Data not shown). Lastly, we confirmed the effect of quercetagetin on cell cytotoxicity by MTT assay. Quercetagetin had no effect on the viability of HaCaT cells at the concentration of 12.5, 25, and 50 μM as shown in Fig. 2C. These result showed that quercetagetin inhibits the TARC and MDC protein level through the down-regulation of gene levels.

Effect of quercetagetin on the STAT pathway in IFN-γ and TNF-α-stimulated HaCaT human keratinocytes

We deduced the action mechanism of quercetagetin on the inhibition of chemokines produced from IFN-γ and TNF-α-stimulated HaCaT human keratinocytes. In the recent reports, IFN-γ and TNF-α-stimulation activates signaling molecules such as STAT, ERK, JNK, p38 MAP kinases, and NF-κB in the HaCaT human keratinocytes (Holvoet *et al.,* 2003). Also, in our recent report, we confirmed the inhibitory effect of EtOH extract of immature *Citrus unshiu* on the phosphorylation of STAT1 induced by IFN-γ and TNF-α-stimulation. Thus, we determined whether quercetagetin affects the STAT pathway in IFN-γ and TNF-α-stimulated HaCaT human keratinocytes using western blot analysis.

First, we confirmed the effect of Jak I, a potent inhibitor of Jaks, on the production of TARC and MDC. Jak I was treated in the IFN-γ and TNF-α-stimulated HaCaT cells and then the effect on production of TARC and MDC was determined using ELISA method. IFN-γ and TNF-α-induced TARC and MDC productions were significantly suppressed by Jak I in a dose-dependent manner. This result exhibits that Jak/STAT pathway participates in IFN-γ and TNF-α-induced TARC and MDC production in HaCaT cells (Fig. 3A).

These results confirmed the pattern of STAT1 phosphorylation in HaCaT cells, stimulation of cells with IFN-γ and TNF-α increased tyrosine 701 phosphorylation of STAT1 at 5 min; the effect peaked at 15 min and then slowly decreased (Data not shown). Treatment with quercetagetin diminished the phosphorylation and the level of STAT1 in a dose-dependent manner (Fig. 3B and C). These observations, taken together with our results, suggest that the action mechanism of quercetagetin might be the regulation of these signal cascades activated by IFN-γ and TNF-stimulation.

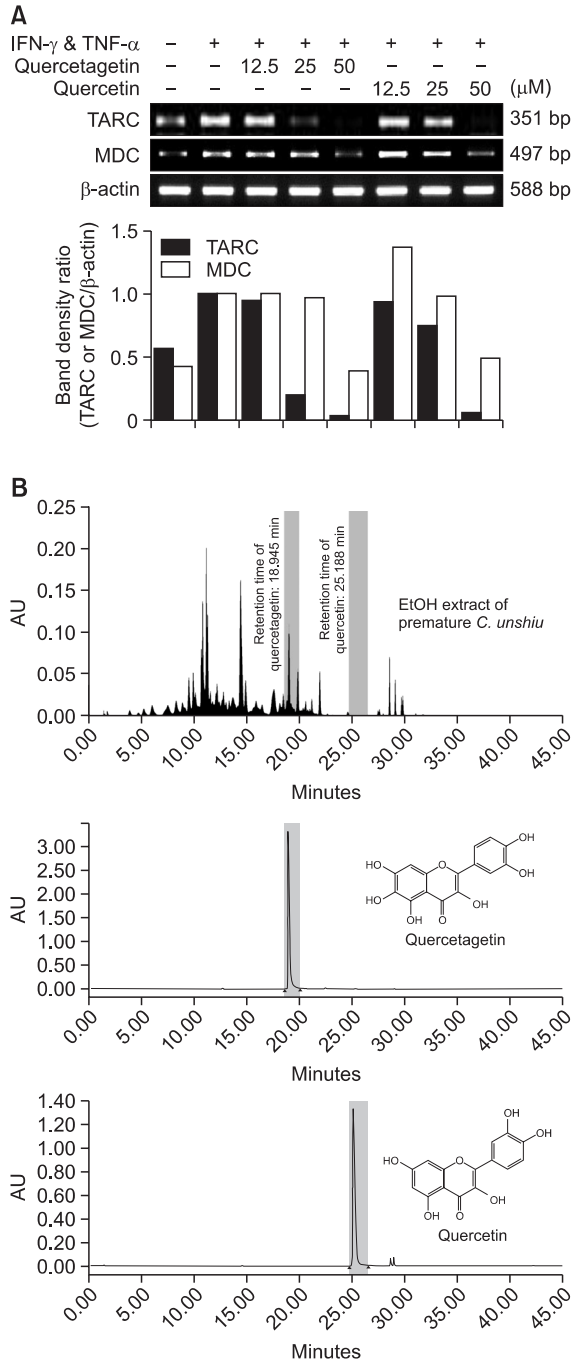

Fig. 4. Comparative Analysis between quercetagetin and quercetin on the TARC and MDC mRNA level in HaCaT human keratinocytes and the existence in immature *Citrus unshiu*. (A) HaCaT Cells were pre-incubated for 18 hr. mRNA expressions of TARC and MDC were measured from the 18 hr culture of cells stimulated by IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) in the presence of quercetagetin and quercetin in a dose-dependent manner (12.5, 25, 50 μM). The mRNA levels were measured by end-point RT-PCR. (B) HPLC analysis of EtOH extract of immature *Citrus unshiu* vs. quercetagetin and quercetin.

Comparison of activity and HPLC analysis between quercetagetin and quercetin

Quercetin is a well-researched flavonoid with broad activity and there are many reports that it affects several inflammatory cytokines and chemokines (Min *et al.,* 2007; Huang *et al.,* 2010; Lee *et al.,* 2010; Panicker *et al.,* 2010). Quercetin and quercetagetin are structurally very similar. Therefore, we next confirmed a difference between two flavonols on TARC and MDC mRNA levels in HaCaT keratinocytes. As a result, quercetagetin showed a stronger inhibitory effect on the IFN-γ and TNF-α-induced mRNA expression of TARC and MDC than quercetin (Fig. 4A).

To confirm whether there is the difference between quercetagetin and quercetin in the peaks of EtOH extract of immature *C. unshiu*, we did high-performance liquid chromatography (HPLC). The standard peaks of quercetagetin and quercetin by HPLC are shown in the middle and bottom of Fig. 4B. Also, HPLC of EtOH extracts of immature *C. unshiu* are shown in the top of Fig. 4B. As results, there is not a matched with the standard peak of quercetin (Retention time of main peak: 25.188 min), while the peak area that matched with the standard peak of quercetagetin (Retention time of main peak: 18.945 min) exists in the peaks of EtOH extract of immature *C. unshiu* (Fig. 4B). Therefore, we concluded that quercetagetin is the main active flavonoid in immature *C. unshiu* that suppresses TARC and MDC production.

DISCUSSION

Citrus plants are a rich source of flavonoids that have a variety of biological activities such as anti-cancer, anti-inflammation, anti-oxidant, anti-diabetes, and anti-allergy effects, *in vitro* and *in vivo* (Silalahi, 2002; Cavia-Saiz *et al.,* 2010). Hesperetin, hesperidin, neohesperidin, naringenin, and naringin are well-researched flavanones. Hesperidin and naringin account for over 90% of bioflavonoids in *Citrus* and have antioxidant, anti-inflammatory and neuroprotective activities (Choi *et al.,* 2007). Nobiletin and tangeretin are specific polymethoxyflavones in *Citrus* with anti-inflammatory and anti-cancer effects (Murakami *et al.,* 2000; Arafa *et al.,* 2009). Also, they have inhibitory effects on chemokine pathways such as monocyte chemoattractant protein-1 (MCP-1) and/or macrophage inflammatory protein-2 (MIP-2) (Lee *et al.,* 2001; Miyata *et al.,* 2011). Quercetagetin and quercitrin are typical flavonols in *Citrus*. Also, quercetagetin has anti-oxidant activity (Yang *et al.,* 2011). However, the components of *Citrus* change according to the harvest date and the immature fruit contains a higher percentage of flavonoids than the mature fruit (Kim *et al.,* 2009). We recently reported that the extract of immature *C. unshiu* inhibits AD related factors *in vitro* and *in vivo* (Kang *et al.,* 2011). Therefore, we investigated the effects of typical flavonoids that are ingredients in *Citrus* on TARC and MDC production. Most flavonoids weakly inhibited or did not inhibit the production of TARC and/or MDC (Fig. 1B). However, quercetagetin strongly repressed the protein and mRNA levels of TARC and MDC without cell cytotoxicity (Fig. 1B and 2). These results show the possibility that quercetagetin might be the active compound of the EtOH extract of immature *C. unshiu* on the inhibition of TARC and MDC production in HaCaT keratinocytes.

We next deduced the action mechanisms of quercetaget-

in on TARC and MDC production. IFN-γ is well known to act via IFNGR1 and R2. Also, activated IFNGRs then activates JAK/STAT, ERK, p38 MAPK, and NF-κB pathways (Gough *et al.,* 2008). We recently reported that the phosphorylation of STAT1 inhibits by the EtOH extract of immature *C. unshiu* (Kang *et al.,* 2011). Hence, we first confirmed the effect of different concentrations of Jak1/2 inhibitor on the TARC and MDC production in IFN-γ and TNF-α-stimulated HaCaT cells. TARC and MDC were reduced through treatment of Jak inhibitors (Fig. 3A). Actually, we confirmed that quercetagetin inhibits the phosphorylation of STAT1 similarly with the effect of EtOH extract of immature *C. unshiu* (Fig. 3B). Based on these results, we guessed that quercetagetin may regulate STAT1 signaling pathways in the production of TARC and MDC by IFN-γ and TNF-α-stimulation.

Lastly, we drew a comparison the difference between quercetagetin and quercetin. Although the structure of quercetagetin is similar to quercetin, the activity of quercetagetin on TARC and MDC mRNA levels is stronger than quercetin (Fig. 4A). On HPLC analysis, the peak for the quercetagetin standard was included in the peaks area for the EtOH extract of immature *C. unshiu*. In contrast, the peak for the quercetin standard did not match the peaks area for the EtOH extract of immature *C. unshiu* (Fig. 4B). Taken together, these results indicate that quercetagetin may be a critical active component of the repression activity of TARC and MDC in immature *C. unshiu*. However, the question on the effect of quercetagetin still remains and a further study may be needed whether the treatment of quercetagetin alone can suppressed the symptoms in AD animal model, like the effect of immature *C. unshiu* extract.

In conclusion, among the major flavonoids in *C. unshiu*, quercetin and quercetagetin strongly suppresses TARC and MDC production in HaCaT human keratinocytes. However, in comparative analysis by HPLC, quercetagetin may be the active compound in immature *C. unshiu*. These results provide new insight into the anti-atopic activities of immature *C. unshiu* and the pharmacological activities that quercetagetin is able to exert on immune modulation, in diseases of excess immune reactions such as AD.

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