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Suppression of thymus- and activation-regulated chemokine (TARC/CCL17) production by 3-*O*-β-D-glucopyanosylspinasterol via blocking NF-κB and STAT1 signaling pathways in TNF-α and IFN-γ-induced HaCaT keratinocytes

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ABSTRACT

A phytosterol derivative, 3-*O*-β-D-glucopyanosylspinasterol (spinasterol-Glc) isolated from leaves of *Stewartia koreana* was reported to inhibit LPS-induced cytokine production in macrophage cells. Thymus and activation regulated chemokine (TARC/CCL17) is produced in response to pro-inflammatory cytokines in keratinocytes, which is implicated in the development of inflammatory skin diseases. In present study, we investigated the effect of spinasterol-Glc on production of TARC/CCL17 induced by TNF-α and IFN-γ in human HaCaT keratinocytes. Spinasterol-Glc inhibited the mRNA and protein expression of TARC/CCL17 induced by TNF-α/IFN-γ in a dose-dependent manner. Inhibitors of c-Raf-1, p38 MAPK, and JAK2, suppressed the TNF-α/IFN-γ-induced production of TARC/CCL17, and phosphorylation of these signaling molecules were attenuated by spinasterol-Glc. The compound also inhibited phosphorylation of IKKα/β and IκB-α, and reduced translocation of NF-κB to the nucleus. We demonstrated that spinasterol-Glc suppressed the NF-κB-driven and the GAS-driven expression of luciferase reporter gene induced by TNF-α and IFN-γ. In addition, spinasterol-Glc inhibited the DNA binding of NF-κB and STAT1 to its cognate binding site. These results suggest that spinasterol-Glc has effective inhibitory effects on production of TARC/CCL17 in keratinocytes via inhibition of NF-κB as well as STAT activation, and could be utilized for development of a potential therapeutic agent against skin inflammatory diseases.

1. Introduction

Keratinocytes play an important function in the pathogenesis of inflammatory skin diseases such as atopic dermatitis (AD). AD is commonly characterized by infiltration of Th2-type lymphocytes into skin lesions and is known to be associated with high levels of chemokines like TARC/CCL17 [1–4]. TARC/CCL17 belongs to a CC chemokine family, attracts CCR4 + Th2 type T cells, and is therefore thought to be related to the development of Th2-mediated inflammatory diseases. It is constitutively expressed in the thymus and keratinocytes, and also produced in other various cell types including endothelial cells and dendritic cells [5–7]. Exposure of keratinocytes to TNF-α and/or IFN-γ induced an abnormal expression of cytokines and chemokines including TARC/CCL17, leading to infiltration of T cells or leukocytes into the site of inflammatory lesion in the skin [1]. It has been reported that TNF-α and IFN-γ stimulate production of TARC/CCL17 synergistically not only in primary human keratinocyte but also in human keratinocyte cell line, HaCaT cells [7,8]. Thus, the downregulation of TARC/CCL17 production in keratinocytes can be effective for treatment of skin diseases.

A variety of herbs and plants have been traditionally used in oriental folk medicine for the treatment of inflammatory diseases. Plant extracts and natural compounds are widely recognized as potential sources of therapeutic agents for prevention and treatment of diseases including inflammatory skin diseases [9–11]. It has been reported that the extracts from *Stewartia koreana* leaves induced angiogenesis, extracellular matrix remodeling in a mouse model, and stimulate wound healing on punched skin of the back of mice [12]. A phytosterol derivative, 3-*O*-β-D-glucopyanosylspinasterol (spinasterol-Glc), was isolated from the extracts of *S. koreana* and was identified as an active compound with strong anti-inflammatory activities [13–15].

The mechanisms of action by which spinasterol-Glc exhibits anti-inflammatory activities in different cell types still remain unclear. In the present study, we investigated the effects of

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spinastol-Glc on production of TARC/CCL17 induced by TNF-α/IFN-γ and the mechanism of its action by which it inhibits the TNF-α/IFN-γ-induced production of TARC/CCL17 in HaCaT keratinocyte cells. We showed that spinasterol-Glc suppressed expression of TARC/CCL17 mRNA and protein induced by TNF-α/IFN-γ. We found that spinasterol-Glc inhibited phosphorylation of Raf1, p38 MAP kinase, JAK2 and STAT1. Furthermore, we demonstrated that spinasterol-Glc inhibited TNF-α/IFN-γ-induced activation of NF-κB and STAT1 and the binding of the factors to the DNA elements contained in TARC/CCL17 promoter.

2. Materials and methods

2.1. Cell culture and reagents

The human keratinocyte HaCaT cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics (100 unit/ml penicillin, 100 μg/ml streptomycin) at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. Dulbecco's modified Eagle's medium (DMEM), FBS, antibiotics, and trypsin–EDTA were obtained from Invitrogen (Carlsbad, CA). Signal inhibitors, BAY11-7082, c-Raf Inhibitor(1-(3-(1,4-dihydroimidazo[4,5-*c*]pyrazol-5-yl)-4-methylphenyl)-3-(3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)phenyl)urea), AG490, PD98059 were obtained from Calbiochem (La Jollla, CA). Specific antibodies against phosphor-p38, phosphor-IκBα, IκBα, p65 and PCNA were obtained from Santa Cruz biotechnology (Santa Cruz, CA). Antibodies against phosphor-JAK2, total JAK2, total p38, phosphor-c-Raf, c-Raf, phosphor Iκκα/β, total Iκκα/β, phosphor STAT1 and total STAT1 were obtained from Cell Signaling Technology (Danvers, MA). HRP-conjugated anti-mouse and anti-rabbit IgG antibodies were obtained from Sigma (St. Louis, MO). Other chemicals were obtained commercially from Sigma Aldrich (St. Louis, MO). The spinasterol-Glc was isolated from the leaves of *S. koreana* as previously described [14]. The structure of spinasterol-Glc is shown in Fig. 1A.

2.2. Cell viability assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were pretreated with spinasterol-Glc in serum-free medium for 1 h and then stimulated with or without 10 ng/ml of TNF-α and IFN-γ (R&D systems, Minneapolis, MN) in the presence or absence of spinasterol-Glc for 24 h. After incubation, cells were treated with 100 μg/ml of MTT for 1 h. The formazan precipitate was dissolved in 200 μl of DMSO and the absorbance at 570 nm was determined by spectrophotometer.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNAs were extracted from the HaCaT cells using Trizol reagent kit (Invitrogen, Carlsbad, CA). cDNA was synthesized from total RNA (2 μg) using M-MuLV reverse transcriptase (Fermentas Life Science, Burlington, Canada). The sequence of primers were as follows: TARC/CCL17 (forward) 5′-ACT GCT CCA GGG ATG CCA TCG TTT TT-3′, (reverse) 5′-ACA AGG GGA TGG GAT CTC CCT CAC TG-3′, β-actin mRNA levels were used as internal controls.

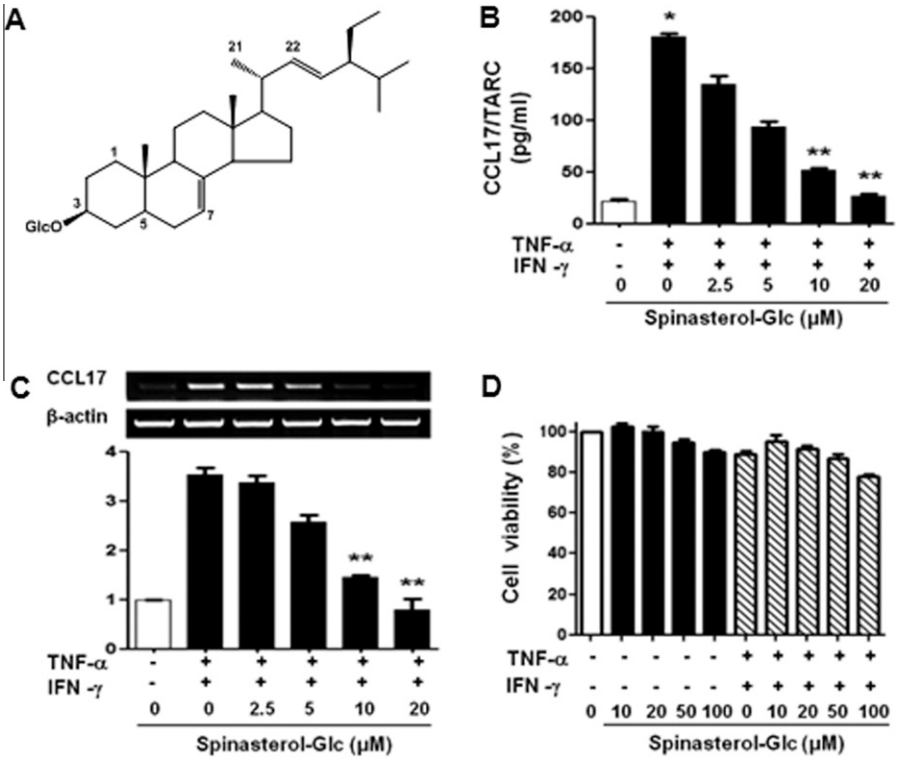

Fig. 1. Effect of spinasterol-Glc on the cell viability and TNF-α/IFN-γ-induced expressions of CCL17 in HaCaT cells. (A) Chemical structure of spinasterl-Glc (3-*O*-*β*-D-glucopyanosylspinasterol). (B) HaCaT cells were pretreated with spinasterol-Glc at the indicated concentration for 1 h, and then exposed to IFN-γ and TNF-α for 18 h. The protein levels of TARC/CCL17 in culture medium were analyzed by ELISA kit according to the manufactures' instruction. Data shown are the average of three independent experiments and are shown as the mean values ± SD. $^{*}p < 0.05$ versus medium alone. $^{**}p < 0.05$ versus TNF-α and IFN-γ-induced cell culture medium. (C) HaCaT cells were treated by the same procedure for ELISA analysis. The levels of TARC/CCL17 mRNAs were determined by RT-PCR analysis and quantified by densitometry. β-actin was used as the internal control. (D) The HaCaT cells were exposed to spinasterol-Glc at the various concentrations for 24 h. Cell viability was measured by MTT assay.

2.4. Enzyme-linked immunosorbent assay (ELISA)

HaCaT cells were cultured in 6 well plates for 24 h and grown in fresh medium containing various concentrations of spinasterol-Glc for 1 h. HaCaT cells were stimulated with 10 ng/ml of TNF-α/IFN-γ for 18 h. The release of chemokine TARC/CCL17 in the culture supernatants were measured by ELISA assay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.5. Western blot analysis

The cells were lysed using RIPA buffer (Pierce Biotechnology, Rockford, IL). The whole cell lysates were electrophoresed on a 8–12% SDS polyacrylamide gel and subjected to Western blot analysis as described previously [16]. Peroxidase-conjugated antibody was used as a secondary antibody. The membrane were developed with an enhanced chemiluminescence system from Amersham and exposed to X-ray film (Fuji Photo Film Co. Ltd.)

2.6. Transient transfection and luciferase assays

Transient transfections were performed using lipofectamine method according to the manufacturer's instructions (Invitrogen, Carsbad, CA). HaCaT cells were transfected with pGAS/Luc or pNF-κB/Luc along with pCis-CK (Stratagene La Jolla, CA), and after 6 h. medium was replaced with growth medium. Transfected cells were grown for 24 h and pretreated with spinasterol-Glc for 1 h, followed by stimulation with TNF-α or INF-γ for 8 h. Cells were harvested and lysed in reporter lysis buffer (Invitrogen, Carlsbad, CA) and luciferase activities were detected using a luciferase assay kit (Invitrogen, Carlsbad, CA). Experiments were repeated at least three times with triplicate samples and values are measured by means ± standard deviations (SD).

2.7. Electrophoretic mobility shift assay (EMSA)

HaCaT cells were pretreated with spinasterol-Glc for 1 h and then stimulated with TNF-α and IFN-γ for 1 h. Preparation of nuclear extracts were performed as described previously [16]. A double-stranded NF-κB-specific deoxyoligomer, 5′-CTCTGGAA-ATCCACAAAC-3′ and STAT-specific deoxyoligomer, 5-TTAGGG-GAACGTGGTTTCC-3′ were used as probes and competitors. Oligomers were annealed and radiolabeled by kination using [γ-^{32}P] ATP (Amersham, USA) and T4 polynucleotide kinase. The reaction products were separated on 6% polyacrylamide gel. The gel was dried and subjected to autoradiography.

2.8. Statistical analysis

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as means ± SD and statistical comparisons between groups were performed using 1-way ANOVA followed by Student's *t*-test.

3. Results

3.1. Spinasterol-Glc inhibited TNF-α/IFN-γ-induced expression of TARC/CCL17 in HaCaT keratinocyte cells

To investigate the effects of spinasterol-Glc on TARC/CCL17 production in TNF-α/IFN-γ-stimulated keratinocyte cells, HaCaT cells were pretreated with various concentrations of spinasterol-Glc for 1 h and then stimulated with TNF-α/IFN-γ for 18 h. TARC/CCL17 production increased markedly up to 186 pg/ml in response to stimulation with TNF-α/IFN-γ, comparing to that of basal level (18 pg/ml). Spinasterol-Glc profoundly inhibited the TNF-α/IFN-γ-induced production of TARC/CCL17 in a dose-dependent manner (Fig. 1B).

In order to determine whether spinasterol-Glc inhibits TARC/CCL17 production at the gene expression level, we investigated the effects of spinasterol-Glc on TARC/CCL17 mRNA levels in TNF-α/IFN-γ-stimulated HaCaT cells. TARC/CCL17 mRNA levels were reduced by spinasterol-Glc concentration-dependently (Fig. 1C). Spinasterol-Glc did not affect cell viabilities of HaCaT at concentrations ranging from 0 to 100 μM for 24 h (Fig. 1D). Our results indicate that spinasterol-Glc inhibits TNF-α/IFN-γ-induced expression of TARC/CCL17 at mRNA levels, which resulted in inhibition of the chemokine production in HaCaT cells.

3.2. Involvement of c-Raf-1, JAK2, and p38 MAPK in TNF-α/IFN-γ-induced expression of TARC/CCL17 in HaCaT keratinocyte cells

NF-κB and STAT1 are known to play pivotal roles in TNF-α/IFN-γ-induced gene expression of cytokines and chemokines including TARC/CCL17 [8–11]. Since the transcription factors are known to be activated by upstream signaling molecules such as c-Raf-1, JAK2 and MAP kinases [16–19], we examined the effects of inhibitors of the signaling molecules on production of TARC/CCL17 in TNF-α/IFN-γ-stimulated keratinocyte cells. HaCaT cells were incubated with inhibitors of c-Raf-1, JAK2 (AG490), ERK MAPK (PD98059), or p38 MAPK (SB203580), or IκBα phosphorylation (Bay11-7082) for 1 h and stimulated with TNF-α and IFN-γ at the concentration 10 ng/ml for 18 h. The amounts of TARC/CCL17 in the cell supernatants were measured by ELISA. The c-Raf-1 inhibitor, SB203580, AG490 and Bay11-7082 inhibited the induction of TARC/CCL17 production by TNF-α/IFN-γ (Fig. 2A), while PD98059 appeared not to inhibit TARC/CCL17 production in HaCaT cells. RT-PCR analysis showed that the inhibitors suppressed expression of TACR/CCL17 mRNA in similar fashion to those of TARC/CCL17 protein levels (Fig. 2B). These results suggest that c-Raf-1, p38 MAPK, and JAK2, but not ERK MAPK are involved in the activation of NF-κB and STAT1 which are required for induction of TARC/CCL17 expression by TNF-α and IFN-γ in keratinocyte cells.

3.3. Spinasterol-Glc inhibited phosphorylation of c-Raf-1, JAK2, p38 MAPK, and IKK activated by TNF-α and IFN-γ in HaCaT keratinocyte cells

To elucidate the molecular basis of inhibitory effects by spinasterol-Glc on the expression of TARC/CCL17 in keratinocytes, we next investigated whether spinasterol-Glc inhibits the activation of Raf-1, p38 MAP kinase, JAK2 and NF-κB signaling event induced by TNF-α and IFN-γ. HaCaT cells were pretreated with spinasterol-Glc for 1 h and then stimulated with TNF-α and IFN-γ for 10 to 30 min. We measured phosphorylation of Raf-1, p38 MAP kinase and JAK2 by Western blot analysis. The results showed that spinasterol-Glc significantly inhibited TNF-α and IFN-γ-induced phosphorylation of Raf-1, p38 MAPK, and JAK2 dose-dependently (Fig. 3A). JAK2 and p38 MAPK are known to be involved in activation of STAT1 [18,19]. Our data showed that spinasterol-Glc attenuated phosphorylation of STAT1 (Fig. 3A).

The pleiotropic NF-κB normally exists in cytoplasm as an inactive complex, the predominant form of which is a heterodimer composed of p50 and p65 subunits by association with inhibitory proteins of the IκB family [17]. Once phosphorylated by the IκB kinase (IKK) complex, IκBs dissociates from the NF-κB subunit and ubiquitinated and rapidly degraded by proteasome [17]. It was previously reported that JAK may contribute to p38 MAPK/NF-κB pathway [20] and c-Raf-1 can activate NF-κB by MEKK1 and IKKβ [16]. IKK phosphorylation significantly increased after being

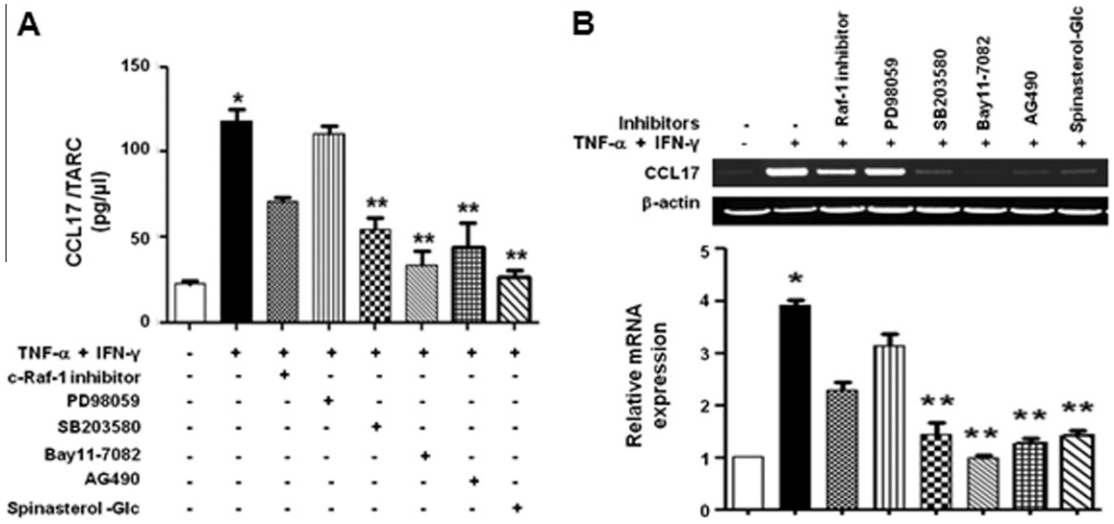

Fig. 2. Effects of various signal inhibitors and spinasterol-Glc on TNF-α and IFN-γ-induced TARC/CCL17 expression in HaCaT cells. (A) HaCaT cells were pretreated with various signal inhibitors, c-Raf inhibitor (50 nM), PD98059 (20 μM), SB203580 (10 μM), Bay11-7082 (10 μM), AG490 (100 μM) and spinasterol-Glc (20 μM) for 90 min and then stimilulated wiith TNF-α/IFN-γ for 18 h. TARC/CCL17 protein levels in the supernatants were detected by ELISA assay. Data shown are the average of three independent experiments and are shown as the mean values ± SD.*$p < 0.05$ versus medium alone. **$p < 0.05$ versus TNF-α and IFN-γ-induced cell culture medium. (B) The levels of TARC/CCL17 mRNAs were determined by RT-PCR analysis and quantified by densitometry. β-actin was used as the internal control.

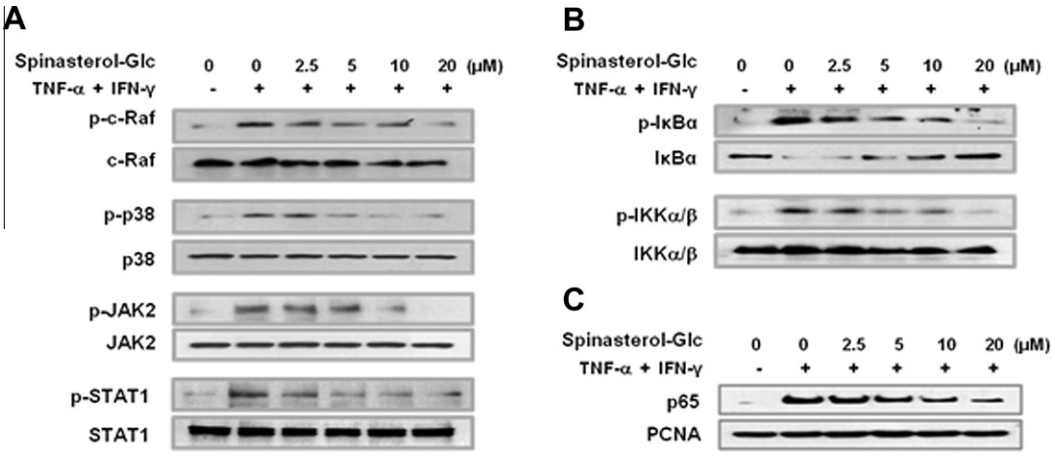

Fig. 3. Effects of spinasterol-Glc on activation of NF-κB and STAT1 signaling molecules and on nuclear translocation of NF-κB. (A) HaCaT cells were pretreated with spinasterol-Glc at the indicated concentration for 1 h and then exposed to TNF-α/IFN-γ. Phosphorylation were determined by a specific antibody against phosphorylated protein. The blots were then stripped and re-probed with antibody against non-phosphorylated protein. (B) HaCaT cells were pretreated with spinasterol-Glc at the indicated concentration for 1 h and then exposed to TNF-α/IFN-γ. Cytosolic proteins were prepared from HaCaT cells and were analyzed by Western blot analysis using an anti-phospho-IkB-α polyclonal antibody and anti-phospho IKK α/β. The blot was stripped and re-probed with antibody against anti-IkB-α and anti-IKK α/β. (C) Nuclear extracts were prepared from HaCaT cells and were analyzed by Western blot analysis using anti-p65. The blot was stripped and re-probed with antibody against PCNA.

stimulated by TNF-α and IFN-γ in HaCaT cells. Pretreatment of the keratinocyte cells with various concentrations of spinasterol-Glc attenuated TNF-α/IFN-γ-induced IKKα/β phosphorylation and nuclear translocation of NF-κB (Fig. 3B and C). Taken together, our results suggest that spinasterol-Glc suppresses expression of TARC/CCL17 by blocking activation of NF-κB and STAT1 via inhibiting phosphorylation of c-Raf-1, JAK2, p38 MAPK, and IKKα/β.

3.4. Spinasterol-Glc inhibited NF-κB and STAT1 activation induced by TNF-α/IFN-γ in HaCaT cells

We next investigated the effects of spinasterol-Glc on TNF-α/IFN-γ-induced transcriptional activation and site-specific DNA binding by NF-κB and STAT1. HaCaT cells were transfected with NF-κB-driven or GAS-driven luciferase construct along with pCis CK negative control plasmid. Addition of TNF-α and IFN-γ induced luciferase activities by 4- to 5-fold, comparing to that of negative control. Spinasterol-Glc suppressed TNF-α/IFN-γ-induced NF-κB and STAT1 activation in HaCaT cells (Fig. 4A and B). These results suggest that NF-κB and STAT1 are activated in response to stimulation with TNF-α and IFN-γ in HaCaT cells, which are essential for expression of TARC/CCL17.

We further examined whether treatment of HaCaT cells with spinasterol-Glc affects TNF-α/IFN-γ-stimulated NF-κB and STAT1 binding to the DNA elements in the TARC/CCL17 promoter. HaCaT cells were treated with spinasterol-Glc for 1 h and co-induced with TNF-α and IFN-γ for 1 h. DNA binding activities of proteins in nuclear extracts of HaCaT cells incubated in the presence or the absence of spinasterol-Glc were analyzed using ^{32}P-labeled oligonucleotides corresponding to the NF-κB site or GAS site (Fig. 4C and D). Formation of NF-κB–DNA complexes or STAT-1–DNA complexes was prominent in nuclear extracts from TNF-α/IFN-γ-stimulated cells.

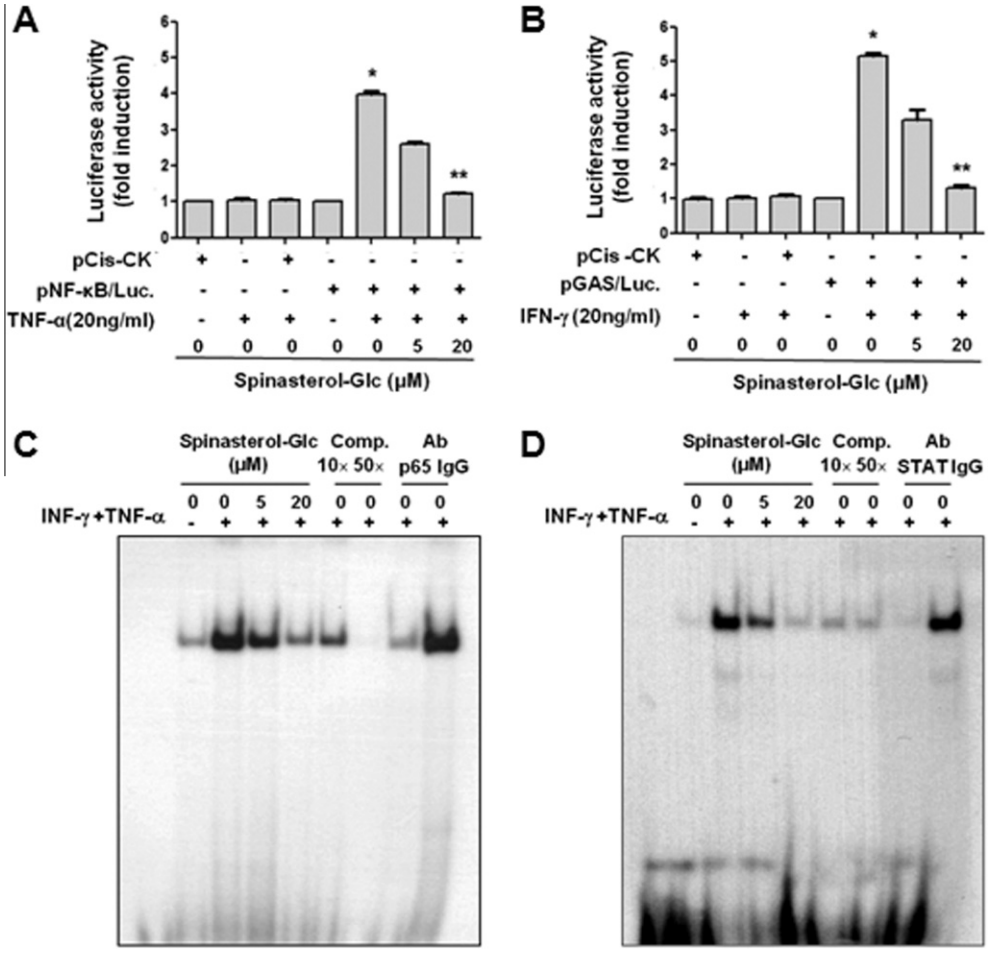

Fig. 4. Effects of spinasterol-Glc on TNF-α and/or IFN-γ-induced in STAT1 and NF-κB promoter activity and DNA binding activity. Cells were transiently transfected with a NF-κB-dependent reporter gene (A) and GAS reporter gene (B). After transfection, cell were incubated for 24 h using growth media, and pretreated with various concentrations of spinasterol-Glc for 1 h and then stimulated with TNF-α and/or IFN-γ- for 8 h. Luciferase activity was determined by the manufacturer's instruction. Data shown are the mean values ± SD ($n = 3$). $^{*}p < 0.05$ compared with negative control values. $^{**}p < 0.05$ versus TNF-α and/or IFN-γ-treated cell control activity values. HaCaT cells were pretreated with spinasterol-Glc for 1 h and then stimulated with TNF-α/IFN-γ for 1 h. The nuclear extracts were analyzed for DNA binding activity to an oligonucleotide containing a specific NF-κB and GAS motif in TARC/CCL17 promoter (C and D). For specific competition, a 10- and 50- fold excess of unlabeled NF-κB and GAS oligonucleotides were added to the reaction mixture.

When HaCaT cells were treated with spinasterol-Glc at 20 μM and stimulated with TNF-α and IFN-γ, the bands of NF-κB–DNA complexes or STAT-1–DNA complexes were markedly reduced comparing to TNF-α/IFN-γ-stimulated cells. Cold oligomer at 50× molar excess inhibited the formation of NF-κB–DNA complexes or STAT-1–DNA complexes almost completely. Antibodies against p65 subunit or STAT1 inhibited the formation of the protein–DNA complexes. These results indicate that spinasterol-Glc inhibited TNF-α/IFN-γ co-stimulated production of TARC/CCL17 by blocking activation of NF-κB and STAT1.

4. Discussion

We have previously demonstrated that spinasterol-Glc suppressed LPS-induced expression of iNOS and cytokine genes such as TNF-α, IL-1β and IL-6 in RAW264.7 murine macrophage cells via inhibiting nuclear translocation and binding of NF-κB to the DNA elements of its target genes [15]. Concomitant with the decreased NF-κB binding, spinasterol-Glc inhibited the activation of ERK1/2, JNK, and p38 MAP kinases, which contribute to the regulation of LPS-stimulated cytokine production in macrophage cells. In the present study, we showed that spinasterol-Glc inhibited production of TNF-α/IFN-γ-stimulated TARC/CCL17 in the HaCaT human keratinocyte cells via inhibiting activation of Raf-1, JAK2, and p38 MAPK, upstream signaling kinases leading to activation of NF-κB and STAT1. We observed that Raf-1, JAK2 and p38 MAPK were rapidly phosphorylated in response to stimulation with TNF-α/IFN-γ in HaCaT keratinocyte cells. It was determined that spinasterol-Glc treatment of keratinocyte cells inhibited TNF-α/IFN-γ-induced activation of the signaling kinases including Raf-1, JAK2, and p38 MAPK as assessed by phosphorylation states.

This study demonstrated that spinasterol-Glc suppresses the induction of TARC/CCL17 protein and mRNA expression in HaCaT cells stimulated with TNF-α and IFN-γ in dose-dependent manner. Spinasterol-Glc dose-dependently inhibits TARC/CCL17 gene expression at transcriptional level. The present data demonstrated that c-Raf-1 was also involved in TNF-α/IFN-γ-induced expression of TARC/CCL17 in HaCaT cells. Raf kinase is known to activate NF-κB via Raf–MEK–ERK cascade by various stimuli such as LPS and TNF-α in different cell types [21]. Previous studies and our results showed that p38 MAP kinase, but not ERK and JNK kinases is involved in TNF-α/IFN-γ-induced expression of TARC/CCL17 in HaCaT cells [11,22,23], indicating that Raf-1 stimulates production of TARC/CCL17 in TNF-α/IFN-γ-stimulated cells in MEK–ERK-independent pathway. Several studies have observed that Raf-1 mediated its effect in the absence of MEK and ERK activation [24,25]. IFN-γ is known to induce the expression of responsive

genes through the activation of JAK and STAT1 transcription factor [18]. Raf-1 was activated by JAK1 in IFN-γ-stimulated COS cells [24], indicating that Raf-1 may be activated by JAK in TNF-α/IFN-γ-treated HaCaT cells in $p21^{ras}$ independent way. We observed that $p21^{ras}$ activity was not changed after treatment with TNF-α/IFN-γ in HaCaT cells (data not shown). Our results showed that spinasterol-Glc inhibited TNF-α/IFN-γ induced phosphorylation of JAK2 and activation of STAT1 in HaCaT cells. In addition, spinasterol-Glc effectively suppressed activation of MEK and ERK, while it did not effectively inhibit activation of JNK (data not shown). MEK and ERK signal molecules are known to activate a number of inflammatory genes such as TNF-α, iNOS and ICAM-1 [21], but the protein kinases appear not to be essential for activation of TARC/CCL17 expression in HaCaT keratinocyte cells. Taken together, our results indicate that spinasterol-Glc can suppress a number of cytokine and chemokine genes including TARC/CCL17 activated by NF-κB and STAT1. Indeed, we observed that spinasterol-Glc inhibited expression of Th1 type chemokines such as CXCL9, CXCL10, and CXCL11 (data not shown).

NF-κB resides in cytoplasm through the association with the IκB proteins, such as IκB-α and IκB-β in unstimulated cells. External stimuli activate IKKs, which in turn phosphorlyate IκBs, leading to the ubiquitination and proteasomal degradation of IκBs [17]. It was suggested that Raf-1 induces IκB degradation independently of MEK and ERK [25], which then stimulate nuclear translocation and binding of NF-κB to its DNA elements. STAT factors have been shown to be phosphorylated by a variety of cytokines and growth factors and participate in the regulation of many genes [18]. STATs are found in cytoplasm before phosphorylation and translocate into the nucleus on activation. STAT1 is known to be activated by JAK or p38 MAP kinase [19] and upon phosphorylation, STAT1 forms homo- or heterodimers with other STATs such as STAT2 and STAT3. Luciferase reporter assays revealed that spinasterol-Glc reduced transcriptional activation of the NF-κB/Luc reporter gene and also GAS/Luc reporter genew in TNF-α/IFN-γ-stimulated HaCaT cells. Gel mobility shift assays showed that spinasterol-Glc inhibited complex formation between NF-κB and its binding sites and also between STAT1 and GAS sequence contained in TARC/CCL17 promoter. Our results demonstrated that TNF-α/IFN-γ-induced activation of NF-κB and STAT1 was effectively attenuated by treatment with spinasterol-Glc in HaCaT cells.

In conclusion, the present study provide envidence that spinasterol-Glc inhibits the expression of TNF-α/IFN-γ-induced expression of TARC/CCL17 gene via suppression of NF-κB as well as STAT1 activation, involving the inhibition of Raf-1, p38 MAP kinase, and JAK2 in HaCaT keratinocyte cells. Our results suggest that spinasterol-Glc may reduce infiltration of Th2 cells into skin lesions by suppressing the interaction between TARC/CCL17 and its receptor CCR4, thereby decrease the disease severity with skin inflammation involved in Th2 type chmokines. Further, spinasterol-Glc could be utilized for the development of a potential anti-inflammatory drug for the treatment of inflammatory diseases like AD.

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