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# Anti-inflammatory Activity of Fucoidan with Blocking NF-κB and STAT1 in Human Keratinocytes Cells

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**Abstract** – Fucoidan, a sulfated polysaccharide is found in several types of edible brown algae. It has shown numerous biological activities; however, the molecular mechanisms on the activity against atopic dermatitis have not been reported yet. We now examined the effects of fucoidan on chemokine production co-induced by TNF-α/IFN-γ, and the possible mechanisms underlying these biological effects. Our data showed that fucoidan inhibited the TNF-α/IFN-γ-induced production of thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) mRNA in human keratinocytes HaCaT cells. Also, fucoidan suppressed phosphorylation of nuclear factor kappa B (NF-κB) and activation of signal transducer and activator of transcription (STAT)1 in a dose-dependent manner. In addition, fucoidan significantly inhibited activation of extracellular-signal-regulated kinases **(**ERK) phosphorylation. These data indicate that fucoidan shows anti-inflammatory effects by suppressing the expression of TNF-α/IFN-γ-induced chemokines by blocking NF-κB, STAT1, and ERK1/2 activation, suggestive of as used as a therapeutic application in inflammatory skin diseases, such as atopic dermatitis.
**Keywords** – Fucoidan, Anti-inflammation, Chemokine, Nuclear factor-kappa B, Signal transducer and activator of transcription 1

## Introduction

Atopic dermatitis (AD) is an important chronic inflammatory disease of the skin, accompanied by itchy eczematous inflammation. While the exact causes of AD are not fully understood, malfunction of the body's immune system and environmental allergenic responses are suggested.[1] Keratinocytes, which are the predominant cell type in the epidermis, are considered to play an important role as a barrier against pathogens. Once transmission of pathogens has occurred in the upper layers of the epidermis, keratinocytes begin to produce pro-inflammatory cytokines and chemokines, leading to pathogenesis of AD.[2]

AD has been associated with development of Th2-mediated inflammatory disease and is related to high levels of chemokine. Thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) belong to the CC chemokine subfamily and are produced by monocyte-derived dendritic cells, endothelial cells, and keratinocytes. TARC and MDC regulate the infiltration of Th2 type lymphocytes to sites of inflammation through the CC chemokine receptor 4 (CCR4) and is a pivotal mediator of the inflammatory reaction of AD. Exposure of keratinocytes to tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) leads to the abnormal expression of pro-inflammatory cytokines and chemokine, such as TARC, MDC, IL-1β, and IL-6. These factors induce the infiltration of inflammatory cells in the skin. It has been reported that stimulation with TNF-α and IFN-γ synergistically increases the production of pro-inflammatory cytokines and chemokine in human keratinocytes.[3-4] Also, it has been demonstrated that serum levels of TARC and MDC are elevated in AD patients, and that increased levels of these chemokine are closely correlated with increased severity of disease.[5-7]

Fucoidan, a sulfated polysaccharide, is found in edible brown algae, such as *Undaria pinnatifida*, *Fucus vesiculosus* and *Ecklonia* cava.[8] It contains various pharmacological principles, including anti-coagulant, anti-thrombotic, anti-tumor, anti-viral, and anti-inflammatory activities.[9-11] Previous reports indicated that fucoidan derived from *Cladosiphon okamuranus* Tokida inhibited the attachment of *Helicobacter pylori* to human gastric cells.[12] It has also been reported that fucoidan suppresses IFN-γ-induced NO/iNOS production in glial cells via inhibition of JAK/

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STAT/IFR-1 and p-p38.[13] Therefore, fucoidan might be useful as a dietary supplement for prevention of human disease, as its chemical structure has no toxic or irritating side effects.[14]

Despite evidence for a number of biological effects of fucoidan, the inflammatory cytokine combination-induced stimulation on human keratinocytes has not been investigated. In this study, we investigated the molecular mechanisms of fucoidan against TNF-α/IFN-γ-induced production of chemokine and cytokines in human keratinocytes, with a particular focus on NF-κB and STAT1 expressions.

## Experimental

**Materials** – Fucoidan and [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). TNF-α and IFN-γ were purchased from R&D Systems (Minneapolis, MN, USA). Primary antibodies against β-actin, IκBα, IKKβ, NF-κB, STAT1 and JAK2 and horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The primary phospho-IκBα, phospho-IKKβ, phospho-NF-κB, phospho-STAT1, phospho-JAK2, phospho-JNK, phospho-p38, phospho-ERK1/2 and JNK, p38, ERK1/2 antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). All other chemicals and reagents were of the highest analytical grade.

**Cell culture** – The human keratinocyte cell line (HaCaT) was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL of streptomycin at 37 ºC in a humidified incubator under a 5% $CO_2$ atmosphere. Cell counts were performed in a hemocytometer from Hausser Scientific (Horsham, PA, USA). For experiments, fucoidan was freshly dissolved in distilled water at concentrations not exceeding 0.01% and was then directly applied to the culture medium.

**Cell viability assay** – The cytotoxic effects of fucoidan against the HaCaT cell line were estimated with a colorimetric assay using the MTT method, which is based on the reduction of a tetrazolium salt by a mitochondrial dehydrogenase in viable cells.[15] Briefly, cells ($2 \times 10^6$ cells/mL) were seeded in 96-well plate and then treated with fucoidan at final concentrations of 1, 5, 10, 30, 50, 70, and 100 μg/mL. After 24 h incubation, MTT solution was added to each well at a final concentration of 0.4 mg/mL. After 2 h of incubation, the supernatants were aspirated and replaced with 150 μL of dimethyl sulfoxide (DMSO) to dissolve the formazan product. The absorbance at 540 nm was read on a spectrophotometric plate reader. Results were calculated as percentages of unexposed control.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)** – Total RNA was isolated from cells using the TRIzol® reagent according to the manufacturer's instructions and quantitated by spectrophotometry. RT-PCR was performed as described previously.[15] Amplification conditions were as follows: 35 cycles of denaturation at 94 ºC for 45 sec, annealing at 55 ºC for 45 sec, and polymerization at 72 ºC for 60 sec. The primer pairs were as follows: TARC sense, 5'-CTTCTCTGCAGCACATCC-3' and TARC antisense, 5'-AAGACCTCTCAAGGCTTTG-3'; MDC sense, 5'-AGGACAGAGCATGGCTCGCCTA CAGA-3' and MDC antisense, 5'-TAATGGCAGGGAG GTAGGGCTCCTGA-3'; IL-1β sense, 5'-AAAAGCTTG GTGATGTCTGG-3' and IL-1β antisense, 5'-TTTCAAC ACGCAGGACAGG-3'; IL-6 sense, 5'-AGAGTAGTGA GGAACAAGCC-3' and IL-6 antisense, 5'-TACATTTG CCGAAGAGCCCT-3'; GAPDH sense, 5'-CCTCTATGC CAACACAGTGC-3' and GAPDH antisense, 5'-ATACT CCTGCTTGCTGATCC-3'. The PCR products were resolved on a 1.2% agarose gel and visualized with UV light after staining with ethidium blotting (EtBr).

**Western blotting analysis** – Western blot analyses were performed as previously described.[16] The cells were cultured, harvested, lysed on ice for 30 min in appropriate lysis buffer (120 mM NaCl, 40 mM Tris (pH 8.0), and 0.1% NP 40) and centrifuged at 13,000 g for 15 min. Lysates from each sample were mixed with 5 × sample buffer (0.375 M Tris-HCl, 5% SDS, 5% β-mercaptoethanol, 50% glycerol, 0.05% bromophenol blue, pH 6.8) and heated to 95 ºC for 5 min. Equal amounts of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membranes were washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membranes were then incubated with respective specific primary antibodies overnight at 4 ºC. After three washes in TBST, membranes were incubated with appropriate secondary antibodies coupled to horseradish peroxidase (HRP) for 1 h at room temperature. The membranes were further washed, and detected by an enhanced chemiluminescence Western blotting detection kit. Data of specific protein levels are presented as fold-change relative to the control.

**Statistical analysis** – All measurements were made in

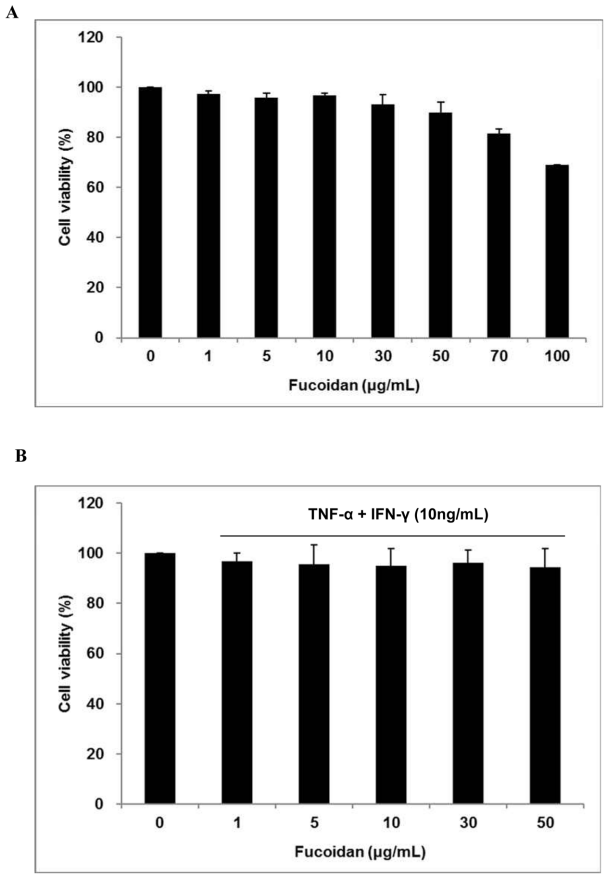


**Fig. 1.** Effects of fucoidan on cell viability of HaCaT cells. (A) Cells were seeded into 96-well plates and treated with various concentrations of fucoidan for 24 h. Cell viability was measured using the MTT assay. (B) Cells were treated with various concentrations of fucoidan and TNF-α/IFN-γ for 24 h. *$p < 0.05$, significantly different from control cells.

triplicate, and all values are given as the mean ± the standard deviation (SD). The results were subjected to an analysis of variance (ANOVA) followed by Tukey's test in order to analyze differences between conditions. In each case, a $p$-value of $< 0.05$ was considered statistically significant.

## Result and Discussion

To determine whether fucoidan can induce cytotoxicity of HaCaT cells, the MTT assay was used. As shown in Fig. 1, cells were incubated with fucoidan at various concentrations ranging from 1 to 100 μg/mL for 24 h. It was demonstrated that, beyond the concentration of 50 μg/mL, no significant change in cell viability was observed. However, fucoidan at 100 μg/mL showed a significant cytotoxic effect on these cells. Thus, we used the concentration of fucoidan at 1 - 50 μg/mL in subsequent experiments.

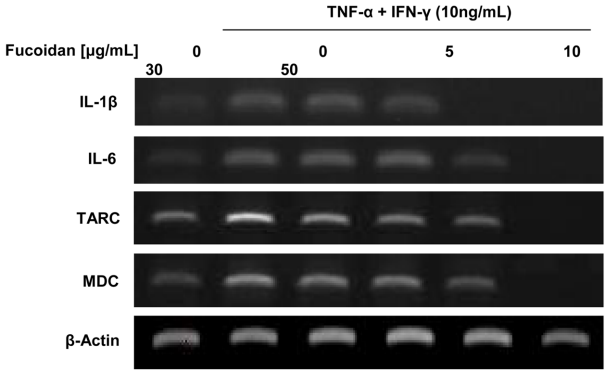


**Fig. 2.** Effects of fucoidan on TNF-α/IFN-γ-induced cytokines and Th2 chemokine expression in HaCaT cells. The mRNA levels of IL-1β, IL-6, TARC, and MDC were analyzed using RT-PCR.

In a previous report, levels of TNF-α/IFN-γ- co-stimulated inflammatory cytokines and chemokine were significantly increased on human keratinocytes. TARC and MDC are designated as Th2 type chemokine, and bind to CCR4+ Th2 cells in inflamed skin. Inflammatory chemokine are expressed in inflamed tissues on stimulation by pro-inflammatory cytokines. IL-1β and IL-6, produced by keratinocytes, are among the best characterized players in the migration of inflammatory cells, keratinocyte proliferation, and the production of other cytokines by keratinocytes.[17] To study whether fucoidan inhibits TNF-α/IFN-γ-induced IL-1β, IL-6, TARC, and MDC expression, HaCaT cells, immortalized human keratinocyte cell line were incubated with TNF-α/IFN-γ (10 ng/mL) in the presence or absence of fucoidan. As shown in Fig. 2, fucoidan significantly inhibited mRNA levels of cytokines (IL-1β and IL-6) and chemokine (TARC and MDC) in TNF-α/IFN-γ-stimulated HaCaT cells in a dose-dependent manner.

Since NF-κB and STAT1 are important regulators of TNF-α/IFN-γ-induced inflammatory responses, we examined the effect of fucoidan on activation of NF-κB and STAT1 in human keratinocytes. NF-κB is important transcription factor that regulates the transcription of a number of inflammatory genes encoding IL-1β and IL-6.[18] NF-κB is located in the cytoplasm and binds to IκB proteins in un-stimulated cells, thereby preventing nuclear entry of both DNA-binding subunits. External stimuli activate IKKs, which in turn mediate ubiquitin-proteasome degradation of IκB in order to prevent the binding of NF-κB to its DNA elements.[19] STAT has also been shown to be phosphorylated by a variety of cytokines and to control the transcription of various genes.[20] STATs are found in cytoplasm before phosphorylation by JAK. The phosphorylated STAT proteins translocate into the cell nucleus,

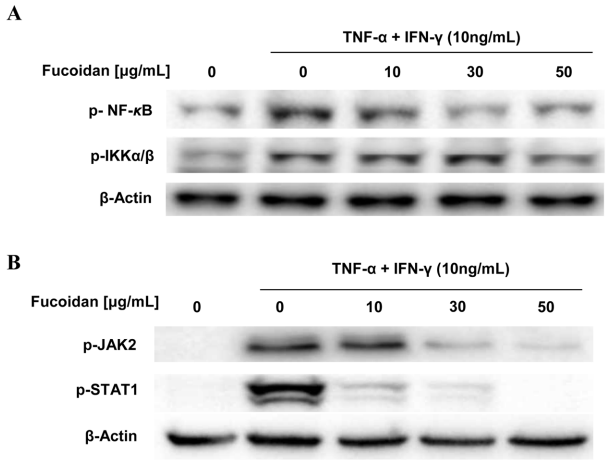


**Fig. 3.** Effects of fucoidan on TNF-α/IFN-γ-induced NF-κB and STAT1 activation in HaCaT cells. Cells were treated with fucoidan, and protein expression was analyzed by Western blotting with (A) phospho-NF-κB and phospho-IKKα/β and (B) phospho-STAT1 and phospho-JAK2 primary antibody.

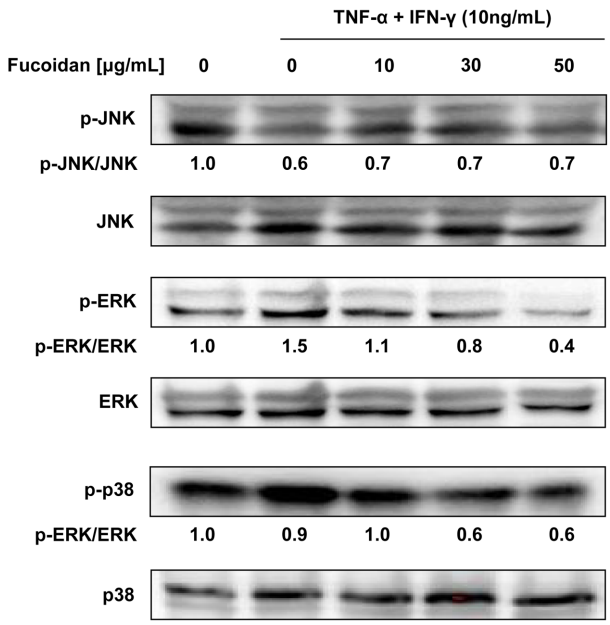


**Fig. 4.** Effects of fucoidan on TNF-α/IFN-γ-induced MAPKs in HaCaT cells. Cells were treated with fucoidan, and total protein lysates were analyzed by Western blotting with ERK, phospho-ERK, p38, phospho-p38, JNK, and phospho-JNK antibodies.

where they regulate the transcription of target genes, including TARC.[21] In our system, cells were stimulated with TNF-α/IFN-γ in the presence or absence of fucoidan at indicated concentrations, and then Western blotting was performed. As shown in Figure 3, fucoidan significantly suppressed the phosphorylation of NF-κB and IKKα/β in a dose dependent manner. In addition, fucoidan inhibited TNF-α/IFN-γ-induced activation of STAT1 and JAK2 in a dose-dependent fashion. Several studies suggested that NF-κB and JAK/STAT are involved in regulation of cytokines and chemokine by TNF-α/IFN-γ stimulation in human keratinocytes.[22-23] Our present results demonstrated that fucoidan suppresses the phosphorylation of NF-κB and STAT1 by TNF-α/IFN-γ stimulation in human keratinocytes. TNF-α/IFN-γ stimulation in human keratinocytes induces the activation of intracellular MAPK signaling pathways. Therefore, to further investigate the involvement of MAPK in TNF-α/IFN-γ-induced activation of NF-κB and STAT1, we examined the activation of p38, JNK, and ERK. Cells were stimulated with TNF-α/IFN-γ in the presence or absence of fucoidan at indicated concentrations, and then Western blotting was performed against phosphorylated MAPK.

As shown in Fig. 4, fucoidan significantly inhibited the phosphorylation of ERK induced by TNF-α/IFN-γ in a dose dependent manner, whereas levels of p38 and JNK phosphorylation were unchanged. Previous studies demonstrated that NF-κB and ERK play a role in involvement of TNF-α/IFN-γ-induced expression of cytokines in keratinocytes.[18] It suggests that fucoidan suppresses the production of TNF-α/IFN-γ-induced cytokines and chemokine production through ERK signaling pathways in human keratinocytes.

In conclusion, our experimental findings provide adequate scientific evidence for traditional use of fucoidan in the treatment of inflammatory diseases. We demonstrate that fucoidan inhibits the expression of IL-1B, IL-6, TARC, and MDC in TNF-α/IFN-γ-induced HaCaT cells. It seems that fucoidan-induced suppression of cytokines and chemokine is mediated by several combined inhibitory effects of important signal pathways including those of NF-κB, STAT1, and ERK. These results suggest that fucoidan may be used as a promising preventive agent through the reduction of infiltration of Th2 cells into skin inflammation by suppressing TARC and MDC production. This is the first report to demonstrate the inflammatory effect of fucoidan on human keratinocytes and to provide a possible mechanism for anti-inflammation activity. Therefore, it is important to develop a dietary/medical supplement using fucoidan in order to suppress the expression of inflammatory cytokines and chemokine.

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