Background: This study investigated the anti-inflammatory effects of bee venom (BV) through the inhibition of nuclear factor kappa beta (NF-κB) expression in macrophages and keratinocytes.

Methods: Cell viability assays were performed to investigate the cytotoxicity of BV in activated macrophages [lipopolysaccharide (LPS)] and keratinocytes [interferon-gamma/tumor necrosis factor-alpha (IFN-γ/TNF-α)]. A luciferase assay was performed to investigate the cellular expression of NF-κB in relation to BV dose. The expression of NF-κB inhibitors (p-IκBα, IκBα, and p50 and p65) were determined by Western Blot analysis, and the electromobility shift assay. A nitrite quantification assay was performed to investigate the effect of BV, and NF-κB inhibitor on nitric oxide (NO) production in macrophages. In addition, Western Blot analysis was performed to investigate the effect of BV on the expression of mitogen-activated protein kinases (MAPK) in activated macrophages and keratinocytes.

Results: BV was not cytotoxic to activated macrophages and keratinocytes. Transcriptional activity of NF-κB, and p50, p65, and p-IκBα expression was reduced by treatment with BV in activated macrophages and keratinocytes. Treatment with BV and an NF-κB inhibitor, reduced the production of NO by activated macrophages, and also reduced NF-κB transcriptional activity in activated keratinocytes (compared with either BV, or NF-κB inhibitor treatment). Furthermore, BV decreased p38, p-p38, JNK, and p-JNK expression in LPS-activated macrophages and IFN-γ/TNF-α-activated keratinocytes.

Conclusion: BV blocked the signaling pathway of NF-κB, which plays an important role in the inflammatory response in macrophages and keratinocytes. These findings provided the possibility of BV in the treatment of atopic dermatitis.

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Introduction

Atopic dermatitis (AD) is a chronic and relapsing skin disorder characterized by cutaneous inflammation and defects in epidermal barrier function [1]. It is one of the most common skin disorders, estimated to be present in up to 1% to 3% of adults, and 20% of children worldwide [2]. Although the pathophysiology of AD remains unclear, epidermal barrier dysfunction due to immunologic responses and genetic defects, play a central role in the aggravation and development of AD [1].

Human skin contains several immunocytes that can be activated by skin damage or invading pathogens. One of the most important immunocytes involved in wound healing and inflammation is the macrophage, which exhibits different immunological functions in the skin, including antigen presentation and phagocytosis. Furthermore, macrophages produce many chemokines and cytokines that stimulate fibrosis, collagen synthesis, and new capillary growth [3,4]. Additionally, keratinocytes which form major structural components of the epidermis also participate in the regulation and/or initiation of cutaneous immune responses and inflammation because of their ability to produce a variety of chemokines and cytokines [5].

Lipopolysaccharides (LPS) are the major components of the external membrane of Gram-negative bacteria, and are one of the most potent initiators of inflammation. LPS activates monocytes and macrophages to produce proinflammatory cytokines [6].

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The mitogen-activated protein kinase (MAPK) family consists of ERK, p38, and JNK [7]. MAPKs are serine-threonine kinases that mediate intracellular signaling associated with various cellular activities including cell proliferation, differentiation, cell death, and inflammation [8].

Nuclear factor-kappa beta (NF-κB) regulates the transcription of numerous genes involved in immunity, inflammation, and protection from programmed cell death. The activation of NF-κB is mediated by various upstream protein kinases, including MAPKs [9]. p50 and p65 constitute NF-κB and are bound to inhibitory IkB proteins in the cytoplasm [10]. The cytoplasmic NF-κB/IκB complex is activated by phosphorylation [11]. A free p50/p65 NF-κB complex translocates from the cytosol to the nucleus, and ultimately binds to the promoter region of target genes encoding various proinflammatory factors [12].

Bee venom (BV) has been used in traditional medicine to treat various inflammatory conditions. BV and its major component, melittin, are potential means of reducing excessive immune responses and provide new alternatives for the control of inflammatory diseases [13,14].

Therefore, we investigated whether BV showed AD related anti-inflammatory effects by inhibiting the activity of NF-κB in activated macrophages and keratinocytes.

Materials and Methods

Materials

The composition of BV (You-Miel Bee Venom Ltd., Hwasoon, Korea) was as follows: 45% to 50% melittin, 2.5% to 3% mast cell degranulating peptide, 12% phospholipase A2, 4% to 5% 6-pentyl a-pyrone lipids, 1% hyaluronidase, 2% to 3% amine, 4% to 5% carbohydrate, and 19% to 27% of other substances, including protease inhibitors, glucosidase, invertase, acid phosphomonoesterase, norepinephrine, dopamine, and unknown amino acids, with 99.5% purity.

All secondary antibodies used in the Western Blot for p50, p65 and p-IκBα, IκBα, β-actin, histone H1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and JNK, p-JNK, p38, p-p38 (Cell signaling Technology, Beverly, MA, USA) and horseradish peroxidase-labeled donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were used at 1:1000. T4 polynucleotide kinase (Promega, Madison, WI, USA) was used for end labelling in the ligation process. Poly (dl-dC) was used as a competitor for nonspecific DNA binding proteins (Amersham Pharmacia Biotech) with the enhanced chemiluminescent (ECL) detection reagent (Amersham Pharmacia Biotech). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) was used to separate the proteins.

Cell culture

The macrophage cell line (Korea Cell Line Bank, Seoul, Korea) were grown at 37°C in Dulbecco's Modified Eagle Medium [DMEM (Gibco, Life Technologies, Grand Island, NY, USA)] supplemented with 10% fetal bovine serum [FBS (Gibco, Life Technologies)], penicillin [100 units/mL (Gibco, Life Technologies)] and streptomycin sulfate (100 µg/mL, Gibco, Life Technologies) in a humidified atmosphere of 5% CO₂. Cells were incubated with BV at various doses (5, 10, or 20 µg/mL) or positive chemicals and then activated with LPS (1 µg/mL) for the indicated time in figure legends. Various doses of BV dissolved in dimethyl sulfoxide (DMSO)-ethanol solvent (1:1 DMSO: ethanol, v/v) were added together with LPS, to the cells. The final concentration of DMSO-ethanol solvent used was less than 0.05%. Cells were treated with 0.05% DMSO-ethanol solvent as a vehicle control.

The keratinocytes were grown in RPMI (Roswell Park Memorial Institute) 1640 (Gibco Life Technologies) with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL) at 37°C in a 5% CO₂ humidifier. All cells were cultured in 24-well plates (Corning Costar) in Minimum Essential Medium (MEM) alpha (Gibco Life Technologies).

Cell viablity assay

To determine the number of viable cells, macrophages and keratinocytes were seeded onto 24-well plates (5 × 10⁴ cells/well) and when the cells became sub confluent they were treated with BV (0-20 µg/mL) for 24 hours. Following treatment cells were trypsinized, pelleted by centrifugation for 5 minutes at 1500 rpm, resuspended in 10 mL of phosphate-buffered saline (PBS), and 0.1 mL of 0.2% trypsin blue was added to the cell suspension (0.9 mL each). Subsequently, a drop of suspension was placed in a Neubauer chamber and the cells were counted. Cells that showed signs of trypsin blue uptake were considered dead, whereas those that excluded trypsin blue were considered viable. Assays were carried out in triplicate.

Transfection and NF-κB luciferase activity

Macrophages and keratinocytes were plated onto a 24-well plates and transiently transfected with a pNF-κB-Luc plasmid (5 × NF-κB, Stratagene, La Jolla, CA, USA) or inducible nitric oxide synthase-luciferase reporter plasmid (5 × NF-κB, Stratagene, La Jolla, CA, USA) or a p50 (C62S) mutant plasmid (5 × NF-κB, Stratagene, La Jolla, CA, USA) using a mixture of plasmid and Lipofectamine Plus in OPTI-MEN (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specification. Cells were transiently co-transfected with a pEGFP-C1 vector (Clontech, Palo Alto, CA, USA) with a WelFect-EX™ PLUS transfection reagent (WelGENE Inc., Daegu, Korea) according to the manufacturer's instructions. After 24 hours of transfection, expression of the green fluorescent protein (GFP) was detected by fluorescence microscopy (DAS microscope: Leica Microsystems, Inc., Deefield, IL, USA). The transfection efficiency was determined as the number of GFP-expressing cells divided by the total number of cells counted, × 100. The transfected macrophages were treated with LPS (1 µg/mL) and different doses (1, 2, and 5 µg/mL) of BV for 8 hours, and the transfected keratinocytes were treated with interferon-gamma/tumor necrosis factor-alpha (IFN-γ/TNF-α) (20 ng/mL) and different doses (1, 2.5, and 5 µg/mL) of BV for 8 hours. Luciferase activity was measured by using the luciferase assay kit (Promega, Madison, WI, USA), and the results were read on a luminometer as described by the manufacturer's specifications (WinGlow, Bad Wildbad, Germany).

Western Blot analysis

Cells were homogenized with a lysis buffer [50 µM Tris, pH 8.0, 150 µM NaCl, 0.02% NaN₃, 0.2% SDS, 1 µm phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 1% igapel 630 (Sigma), 10 µM NaF, 0.5 µM EDTA, 0.1 µM EGTA, and 0.5% sodium deoxycholate] and centrifuged at 23,000 g for 1 hour. Equal amounts of proteins (80 µg/lane) were separated on SDS-12% polyacrylamide gels and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech). Blots were blocked for 2 hours.
at room temperature with 5% (w/v) nonfat dried milk in Tris buffered saline (10 µM Tris, pH 8.0, 150 µM NaCl) containing 0.05% Tween 20. The membrane was incubated for 5 hours at room temperature with the following specific antibodies: Rabbit polyclonal antibodies against p50, p65 and p-IκBα, IκBα, β-actin, Histone H1 (1:1,000), and mouse monoclonal antibodies directed against JNK, p-JNK, p38, p-p38 (1:1,000) were used in the study. The blot was then incubated with the corresponding conjugated anti-rabbit and anti-mouse immunoglobulin G-horseradish peroxidase. Immunoreactive proteins were detected with the ECL Western Blot detection system.

**Electromobility shift assay**

The DNA binding activity of NF-κB was determined using an electromobility shift assay (EMSA) performed as according to the manufacturer’s recommendations (Promega, Madison, WI, USA). Nuclear extracts were prepared and processed for EMSA. The relative densities of the DNA-protein binding bands were scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified.

**Nitrite quantification assay**

Nitric oxide (NO) was determined through the estimation of nitrite level in the cell culture media. The nitrite accumulation in the supernatant was assessed by Griess reaction. The macrophages were seeded into 6-well plates (1×10^6 cells/well) with 2 mL of cell culture media, and incubated for 24 hours. The old culture media was discarded and replaced with new media to maintain the cells. Equal doses of BV (5 µg/mL) and NF-κB inhibitor (5 µM) were used to pretreat the macrophages. The macrophages were activated with LPS (1 µg/mL) with the exception of the control group, for a further 24 hours. A 100 µL sample of the supernatants was added to 100 µL of Griess reagent (0.1% NED, 1% sulphanilamide, and 2.5% phosphoric acid) and incubated at room temperature for 10 minutes in the dark. The absorbance was determined by using a microplate reader (ThermoFisher Scientific, Seoul, Korea) at 540 nm. The NO concentration was determined by comparison to the standard curve.

**Data analysis**

The data were analyzed using the GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA) and presented as mean ± SD. Differences in the data were assessed by a 1-way analysis of variance (ANOVA). When the p value in the ANOVA test indicated statistical significance, the means were separated with a Dunnett’s post hoc test. A value for p < 0.05 was considered to be statistically significant.

**Results**

**Cytotoxicity of BV on Cell Growth in Macrophages**

To assess the cytotoxicity of BV on macrophages, cell viability was analyzed and recorded as the percentage of viable cells. The cells were treated with several doses of BV (1, 2.5, 5, 10, and 20 µg/mL) for 24 hours. As shown in Fig. 1, BV did not inhibit cell proliferation of the macrophages. Morphological observations showed no significant difference between the control and the BV treated macrophages. Therefore, BV (1 µg/mL to 20 µg/mL) was not cytotoxic for macrophages.

**Inhibitory Effects of BV on NF-κB dependent luciferase in LPS-induced macrophages**

To investigate the role of BV in NF-κB dependent gene transcription, a transient transfection assay was conducted with a fusion gene containing an SV40 promoter, 5 repeats of the consensus NF-κB-binding sequence, and the luciferase reporter gene. Macrophages were transfected with this promoter-reporter gene construct, and transcriptional activities were measured after activating the macrophages with LPS (1 µg/mL), with or without BV (1, 2, and 5 µg/mL). Luciferase activity of NF-κB in activated macrophages was reduced with increasing doses of BV (Fig. 2).

**Inhibitory effects of BV on LPS-induced NF-κB activation in Macrophages**

To investigate whether BV is able to inhibit DNA-binding activity of NF-κB in LPS-activated macrophages, nuclear extracts were prepared and assayed for NF-κB DNA-binding activity by EMSA (Fig. 3). LPS significantly induced NF-κB; however, its activity was blocked by BV. Translocation of IκB and p-IκB in the cytosol of activated macrophages and translocation of p50 and p65...
in the nucleus of the cells was inhibited by BV in a dose-dependent manner (Fig. 4).

**Synergistic effects of BV and NF-κB inhibitor on the concentration of NO in macrophages**

To confirm whether an NF-κB inhibitor exerts cytotoxic effects on macrophages through the NO pathway, the NO contraction in the supernatant was assessed by Griess reaction following treatment with an NF-κB inhibitor (5 μM) or/and BV (5 μg/mL). In comparison with the control group, the level of NO was reduced by the complex treatment group (NF-κB inhibitor and BV), as compared with the single treatment group. Therefore, it was confirmed that the complex treatment group of NF-κB inhibitor and BV had a synergistic effect on inhibition of NO production (Fig. 5).

**Inhibitory effects of BV on MAP kinase pathway in activated macrophages**

To investigate the involvement of the MAP kinase pathway on the inhibitory effect of BV on NO production, the activation of MAP kinase (phosphorylation of JNK and p38) in activated macrophages was evaluated. The Western Blot analysis individually from 3 different experiments showed that BV (1, 2.5 and 5 μg/
mL) strongly blocked activation of p-JNK and p-p38 in activated macrophages (Fig. 6). These results suggest that BV inhibits the activation of p-JNK and p-p38 and blocks the MAPK pathway leading to inactivation of NF-κB in activated macrophages.

**Cytotoxicity of BV on cell growth in keratinocytes**

To assess the cytotoxicity of BV on cell growth of keratinocytes, cell viability was analyzed as the percentage of viable cells. The cells were treated with several doses of BV (1, 2.5, 5, 10, and 20 µg/mL) for 24 hours. As shown in Fig. 7, BV (20 µg/mL) moderately inhibited cell proliferation of keratinocytes in a dose-dependent manner. However, no significant variation in cell viability existed after treatment with BV doses between 1 µg/mL to 10 µg/mL. In fact, cell viability improved moderately as compared with the control. Morphologically, the cells appeared smaller in size after treatment with BV (20 µg/mL, Fig. 7). It was found that BV does not become cytotoxic within the dose range of 1 µg/mL to 5 µg/mL of BV.

**Inhibitory Effects of BV on NF-κB dependent Luciferase in IFN-γ/TNF-α activated keratinocytes**

To investigate the role of BV in NF-κB dependent gene transcription, a transient transfection assay was conducted with a fusion gene containing SV40 promoter, 5 repeats of the consensus NF-κB-binding sequence, and the luciferase reporter gene. Keratinocytes were transfected with this promoter-reporter gene construct, and transcriptional activities were measured after activating the cells with IFN-γ/TNF-α (20 ng/mL) with or without BV (1, 2.5, and 5 µg/mL). As shown in Fig. 8, treatment of BV in the transfected cells inhibited the luciferase activity induced in activated keratinocytes (Fig. 8).

**Inhibitory effect of BV on NF-κB in IFN-γ/TNF-α activated keratinocytes**

To investigate whether BV is able to inhibit NF-κB in IFN-γ/TNF-α activated keratinocytes, a Western Blot analysis was performed. NF-κB activation was significantly increased in IFN-γ/TNF-α activated keratinocytes (Fig. 1).
TNF-α-activated keratinocytes; this was blocked by BV. Inhibition by BV was dose-dependent for the translocation of IκB and p-IκB in the cytosol and p50 and p65 in the nucleus of IFN-γ/TNF-α-activated keratinocytes (Fig. 9).

Synergistic effects of BV and the NF-κB inhibitor on NF-κB activity in keratinocytes

To investigate whether BV could inhibit NF-κB in keratinocytes treated with LPS (1 µg/mL), the NK-κB luciferase assay was performed. Both the BV treatment group and the NF-κB inhibitor treatment group inhibited the activity of NF-κB in keratinocytes treated with LPS. In addition, in the groups treated with BV and NF-κB inhibitor, the activity of NF-κB in keratinocytes was suppressed more than in the single treatment groups (Fig. 10). Therefore, a synergistic effect of BV and NF-κB inhibitor on NF-κB activity in keratinocytes was identified.

Inhibitory effects of BV on MAP kinase pathway in IFN-γ/TNF-α-activated keratinocytes

MAP kinase (phosphorylation of JNK and p38) induced in IFN-γ/TNF-α-activated keratinocytes was investigated for the potential inhibitory effects of BV on NO production in the MAPK pathway. Western Blot analysis from 3 individual experiments showed that BV (1, 2.5, and 5 µg/mL) blocked p-JNK and p-p38 in IFN-γ/TNF-α (20 ng/mL)-activated keratinocytes (Fig. 11). These results suggest that BV inhibits the activation of p-JNK and p-p38 in IFN-γ/TNF-α-induced keratinocytes and blocks the MAPK pathway leading to inactivation of NF-κB.
Discussion

AD is one of the most common and intensively studied chronic inflammatory skin disorders [15,16]. Several cofactors, such as an impaired skin barrier function, modifications of the immune system, and a complex genetic background, direct the course of AD [17-19]. Within this complex network, macrophages play a pivotal role in enhanced susceptibility to cutaneous infections, and act as central connecting components in the pathogenesis of AD on the cellular level [20]. In AD, macrophages are known to accumulate in acutely and chronically inflamed skin [21]. During the early, and short inflammatory phase, macrophages exhibit proinflammatory functions such as antigen-presenting phagocytosis, production of inflammatory cytokines and growth factors that facilitate the resolution of inflammation [3,22]. The activation of these signaling pathways by an inflammatory response leads to the synthesis of proinflammatory chemokines and cytokines. In vitro experiments with TNF-α and/or IFN-γ-activated keratinocytes have shown that these signaling pathways were activated resulting in an increased expression of inflammatory chemokines and cytokines [23-26].

BV, which is a complex mixture of active peptides, has long been used in China, Japan, and Korea as a traditional medicine. It contains melittin, phospholipase A2, apamin, adolapin, and mast cell degranulating peptides [14]. Recent studies have shown that treatment with BV can induce a significant anti-inflammatory response via inflammatory mediators, similar to what is achieved with the administration of non-steroidal anti-inflammatory drugs [27-29].

Several studies have reported the effects of BV on AD. Sur et al [30] reported that injection with BV at BL40 effectively alleviates AD-like skin lesions by inhibiting inflammatory and allergic responses in a trimellitic anhydride-induced contact hypersensitivity mouse model. An et al [31] reported that BV and melittin had ameliorating effects on dinitrochlorobenzene-induced AD-like skin lesions of Balb/c mice and TNF-α /IFN-γ-activated human keratinocyte HaCaT cells. Jung et al [32] reported that BV phospholipase A2 (bvPLA2) ameliorates house dust mite-induced AD-like skin lesions in mice by suppressing the expression of inflammatory chemokines and cytokines [33].

In a similar manner, the luciferase assay was performed (instead of the Nitrite quantification assay) in IFN-γ/TNF-α-activated keratinocytes treated with BV and showed an anti-inflammatory effect through the inhibition of NF-κB activity.

In conclusion, BV reduced the expression and transcriptional activity of NF-κB, which plays an important role in inflammatory responses in macrophages and keratinocytes. Although further in vivo and in vitro studies (using a squamous cell line) are needed to substantiate the current work, these findings provide new insight into the application of BV in the preventive intervention for AD.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References