Analysis of the Apoptotic Mechanisms of Snake Venom Toxin on Inflammation–induced HaCaT Cell–line

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[Abstract]

Objectives: In this study, the roles of Interleukin (IL)–4 and Signal transducer and activator of transcription 6 (STAT6), which have been reported to play a role in the pathogenesis of inflammation and cancer, were evaluated in snake venom toxin (SVT)–induced apoptosis.

Methods: Inflammation was induced in human HaCaT keratinocytes, by lipopolysaccharide (LPS; 1 μg/mL) or tumor necrosis factor–α (TNF–α), followed by treatment with SVT (0, 1, or 2 μg/mL). Cell viability was assessed by MTT assays after 24 h, and the expression of levels of IL–4, STAT6, and the apoptosis–related proteins p53, Bax, and Bcl–2 were evaluated by western blotting. Electromobility shift assays (EMSAs) were performed to evaluate the DNA binding capacity of STAT6.

Results: MTT assays showed that inflammation–induced growth of HaCaT cells following LPS or TNF–α stimulation was inhibited by SVT. Western blot analysis showed that p53 and Bax, which promote apoptosis, were increased, whereas that of Bcl–2, an anti–apoptotic protein, was decreased in a concentration–dependent manner in LPS– or TNF–α–induced HaCaT cells following treatment with SVT. Moreover, following treatment of HaCaT cells with LPS, IL–4 concentrations were increased, and treatment with SVT further increased IL–4 expression in a concentration–dependent manner. Western blotting and EMSAs showed that the phosphorylated form of STAT6 was increased in HaCaT cells in the context of LPS– or TNF–α–induced inflammation in a concentration–dependent manner, concomitant with an increase in the DNA binding activity of STAT6.

Conclusion: SVT can effectively promote apoptosis in HaCaT cells in the presence of inflammation through a pathway involving IL–4 and STAT6.

Key words: Snake venom toxin; Inflammation; Apoptosis; Interleukin–4; Signal transducer and activator of transcription 6

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I. Introduction

Keratinocytes are major structural components of the epidermis and participate in the initiation and/or regulation of cutaneous inflammatory and immune responses owing to their ability to produce a variety of cytokines and chemokines.

SVT was previously shown to act as a promising chemotherapeutic agent in the treatment many types of cancer cell, including prostate cancer cells, neuroblastoma cells, and colon cancer cells, through induction of apoptotic cell death mediated by apoptosis regulatory proteins.

Apoptosis of keratinocyte is a key mechanism protecting against squamous cell carcinoma through removal of premalignant cells that have acquired mutations. In the skin, different apoptotic programs are needed to regulate cells other than melanocytes. The relative deficiency of apoptotic inhibitors in keratinocytes may function to maintain a low apoptotic threshold, as is required to sustain rapid turnover and efficiently remove damaged cells.

In patients with atopic dermatitis (AD), the skin produces interleukin (IL)–4, which plays a pivotal role in inducing proliferation and differentiation into T–helper 2 (Th2) cells and promoting IgE production as a major isotype switching regulator.

The known functions of IgE antibodies in allergic inflammation suggest that IgE and IgE–mediated mast cell and eosinophil activation contribute to AD.

Therefore, in present study, the effects of SVT on the inhibition of inflammation and promotion of apoptosis were evaluated in inflammation–induced HaCaT cells, and the roles of IL–4 and signal transducer and activator of transcription 6 (STAT6) in this process were assessed.

II. Materials and Methods

1. Materials

SVT from Vipera lebetina turanica was purchased from Sigma (St, Louis, MO, USA). HaCaT human keratinocytes were obtained from the American Type Culture Collection (Cryosite, Lane Cove NSW, Australia). Lipopolysaccharide (LPS) and tumor necrosis factor (TNF)–α were purchased from Sigma–Aldrich (St, Louis, MO).

The specific antibodies used in western blot analysis were purchased from companies as indicated below. All other reagents were purchased from Sigma unless otherwise stated.

Bcl–2 (1:1,000 dilution) were purchased from Cell Signaling Technology (Beverly, MA), Bax, STAT6 and p–STAT6 (1:1,000 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and IL–4 (1:1,000 dilution) were purchased from Thermo Fisher (Waltham, MA).

2. Cell culture

HaCaT cells were grown in Dulbecco’s modified Eagle’s medium (DMEM: Gibco, Life Technologies, Grand Island, NY) with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO₂ humidified. All cells were cultured in 24–well plates from Costar. RPMI 1640, MEMalpha, penicillin, streptomycin, and FBS were purchased from Gibco Life Technologies (Grand Island, NY).

3. Cell viability assay

To determine viable cell numbers, HaCaT human keratinocytes were seeded onto 24–well plates (5 × 10⁴ cells/well), and subconfluent cells were subsequently treated with snake venom (0–2 μg/mL) for 24 h. The cells were trypsinized,
pelleted by centrifugation for 5 min at 1500 rpm, and resuspended in 10 mL phosphate-buffered saline (PBS). Subsequently, 0.1 mL of 0.2% trypan blue was added to each cell suspension (0.9 mL). Subsequently, a drop of each suspension was placed in a Neubauer chamber, and the number of HaCaT cells was counted. Cells that showed signs of trypan blue uptake were considered to be dead, whereas those that excluded trypan blue were considered to be viable. Each assay was carried out in triplicate.

4. Western blot analysis

Cells were homogenized with lysis buffer (50 μM Tris, pH 8.0, 150 μM NaCl, 0.02% NaN₃, 0.2% sodium dodecyl sulfate [SDS], 1 μM phenylmethylsulfonyl fluoride, 10 μL/mL aprotinin, 1% igapel 630 [Sigma], 10 μM NaF, 0.5 μM ethylenediaminetetraacetic acid [EDTA], 0.1 μM EGTA, and 0.5% sodium deoxycholate) and centrifuged at 23,000 × g for 1 h. Equal amounts of proteins (80 μg) were separated on 12% SDS-polyacrylamide gels and then transferred to a nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech). Blots were blocked for 2 h 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 membranes were then incubated for 5 h at room temperature with the following specific antibodies: Bcl–2 (1:1,000 dilution; Cell Signaling Technology), Bax, STAT6 and p–STAT6 (1:1,000 dilution; Santa Cruz Biotechnology) and IL–4 (1:1,000 dilution; Thermo Fisher). Blots were then incubated with corresponding anti–rabbit and anti–mouse immunoglobulin G– secondary antibodies conjugated with– horseradish peroxidase (1:2,000 dilution; Santa Cruz Biotechnology). Immunoreactive proteins were detected with an ECL western blotting detection system.

5. Electro mobility shift assay (EMSA)

The DNA binding activity of STAT6 was determined using EMSAs (Promega) according to the manufacturer’s recommendations. Nuclear extracts were prepared and processed for EMSA as previously described. The relative densities of the DNA/protein binding bands were scanned by densitometry using MyImage (SLB), and quantified by Labworks 4.0 software (UVP, Inc., Upland, CA).

6. Statistical analysis

The data were analyzed using the GraphPad Prism 4 ver. 4.03 software (Graph–Pad Software, La Jolla, CA). Data are presented as means ± standard deviations (SDs). Differences in all data were assessed by one–way analysis of variance (ANOVA). When the p value obtained from ANOVA indicated statistical significance, the differences were further assessed by Dunnett’s tests. p values of 0.05 or less were considered statistically significant.

III. Results

1. Effects of SVT on the growth of HaCaT cells in the context of LPS–induced inflammation

To assess the inhibitory effects of SVT in the presence of LPS (1 μg/mL) on cell growth of human HaCaT keratinocytes, cell viability was analyzed by MTT assay. The treatment of LPS inhibited the HaCaT cells growth. The cells were treated with two concentrations of SVT (1 and 2 μg/mL) for 24 h in the presence of LPS, SVT augmented LPS–induced inhibition of cell growth of HaCaT cells (Fig. 1).
2. Effects of SVT on the growth of HaCaT cells in the context of TNF-α-induced inflammation

To assess the inhibitory effects of SVT in the presence of TNF-α on cell growth in human HaCaT keratinocytes, cell viability was analyzed by MTT assays. Treatment with TNF-α inhibited HaCaT cell growth. Cells were then treated with two concentrations of SVT (1 or 2 μg/mL) for 24 h in the presence of TNF-α. The results showed that SVT augmented TNF-α-induced inhibition of cell growth in HaCaT cells (Fig. 2).

3. Apoptotic effects of SVT on HaCaT cells in the context of LPS-induced inflammation

To evaluate apoptotic cell death in response to SVT under atopic conditions induced by LPS in vitro, western blot analysis was performed in HaCaT cells. LPS was used to induce atopic conditions in HaCaT cells, Expression levels of IL-4, p53, and Bax were increased, whereas the expression of anti-apoptotic Bcl-2 was significantly decreased follow-

4. Apoptotic effects of SVT on HaCaT cells in the context of TNF-α-induced inflammation

To evaluate apoptotic cell death in response to SVT under atopic conditions induced by TNF-α in vitro, blot analysis was performed in HaCaT cells. TNF-α was used to induce atopic conditions in HaCaT cells, Expression levels of IL-4, p53, and Bax were significantly enhanced, whereas Bcl-2 expression was decreased by the treatment with TNF-α or SVT (1 and 2 μg/mL) in a concentration-dependent manner (Fig. 4).

5. Effects of SVT on LPS-induced STAT6 activation in HaCaT cells

To investigate whether SVT affected LPS-induced STAT6 activation, EMSAs were used to detect the DNA binding activity of STAT6. The results showed that LPS-treated HaCaT cells showed low constitutive activation of STAT6, How-
ever, treatment with SVT enhanced LPS–induced DNA binding activity of STAT6 in a concentration-dependent manner (Fig. 5). Consistent with these findings, the phosphorylation of STAT6 in the nucleus was enhanced by SVT treatment in LPS–induced HaCaT cells (Fig. 5).

### 6. Effects of SVT on TNF–α–induced STAT6 activation in HaCaT cells

To investigate whether SVT affected TNF–α–induced STAT6 activation, EMSAs were performed to
IV. Discussion

In this study, the results showed that SVT was effective for reducing cell growth in human HaCaT cells under inflammatory conditions based on the observation that SVT inhibited the growth of inflammation–induced HaCaT cells, enhanced cytotoxicity through the increased expression of IL–4 and STAT6, and activated apoptosis by regulating the activities of pro–apoptotic p53, pro–apoptotic Bax, and anti–apoptotic Bcl–2 simultaneously.

LPS has been shown to inhibit the extracellular membrane of gram–negative bacteria. As an endotoxin, LPS stimulates the expression of inducible nitric oxygen synthase (iNOS), which produces inflammatory cytokines, such as TNF–α, IL–6, and IL–1, as well as the inflammatory mediator nitric oxide (NO), in macrophages. In particular, TNF–α is secreted as an important stimulator of for the early inflammatory response and then induces the expression of IL–6 and IL–8, leading to a series of inflammatory responses.

IL–4 has been shown to induce IgE production in B cells and to be secreted by Th2 cells. Moreover, IL–4 plays a key role in allergic inflammation. Divergent effects of IL–4 have been reported in hematopoietic cells, fibroblasts, and epithelial cells. In the monocyte/macrophage lineage, IL–4 displays anti–inflammatory properties, e.g., inhibition of LPS–induced IL–1β, TNF–α, and IL–10 production. In particular, IL–4 regulates TNF–α–induced transcription of IL–8, a CXC chemokine induced by pro–inflammatory cytokines, such as TNF–α, IL–1β, and IL–6, in different cell types, including keratinocytes; this mechanism involves STAT6 and nuclear factor (NF)–κB. Binding of IL–4 to its receptor leads to activation of the Janus kinase (JAK)/STAT signal transduction pathway. IL–8 signaling increases the proliferation and sur–
vival of endothelial and cancer cells and promotes the migration of cancer cells, endothelial cells, and infiltrating neutrophils at the tumor site. Therefore, inhibiting the effects of IL-8 signaling may be an important therapeutic intervention in the targeting of the tumor microenvironment.

The tumor-suppressor p53 is an anticancer gene that functions to regulate Bcl-2 and Bax gene expression in vitro and in vivo, thereby inhibiting abnormal cell proliferation and inducing the death of cancer cells through modulation of apoptosis, via regulating the ratio of pro-apoptotic bax and anti-apoptotic bcl-2, a vital tumor suppressive process. The most intuitive link between p53-mediated transactivation and apoptosis is based on its ability to control the transcription of pro-apoptotic members of the Bcl-2 family, including Bcl-2, Bax, and Bak: their net effect is to increase the ratio of pro-apoptotic to anti-apoptotic Bcl-2 proteins, thereby favoring the release of apoptogenic proteins from the mitochondria, promoting caspase activation, and enhancing apoptosis.

AD-like conditions, including inflammation, were induced by LPS or TNF-α in this study. The results showed that IL-4 inhibited TNF-α-induced IL-8 through activation of the JAK/STAT pathway in HaCaT cells and that SVT significantly inhibited LPS- or TNF-α-induced inflammation in HaCaT cells in a concentration-dependent manner compared with that in the control (Figs. 1, 2). The expression levels of IL-4, p53, and Bax were significantly increased in LPS- and TNF-α-induced keratinocytes depending on the concentration of SVT, whereas Bcl-2 expression was decreased (Figs. 3, 4). In HaCaT cells, expression of apoptotic regulators, such as Bcl-2 and Bax, is absent or barely detectable; however, p53 expression is strong. According to the results of this study, in addition to enhancing the expression of p53, SVT enhanced Bax expression and suppressed Bcl-2 expression, suggesting that SVT promoted apoptosis.

Treatment with SVT enhanced the LPS- and TNF-α-induced DNA binding activity STAT6 in a concentration-dependent manner, and levels of phosphorylated STAT6 were also increased (Figs. 5, 6). These data indicated that IL-4 was associated with STAT6 and that SVT promoted the apoptosis of HaCaT cells under inflammatory conditions.

In conclusion, the effects of SVT on inflammatory keratinocytes were evaluated in vitro, and the results showed that SVT could promote apoptosis, causing rapid turnover and removing damaged cells in the skin. Although further studies using squamous cell lines in vitro or animal experiments in vivo are needed to confirm the results of this study, these findings provide new insights into the application of SVT as a preventive intervention in the management of squamous cell carcinoma.

### V. References

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