Original Article
Shikonin inhibits IFN-γ-induced K17 over-expression of HaCaT cells by interfering with STAT3 signaling

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Abstract: Objectives: We hypothesized that interferon-γ (IFN-γ) induces K17 over-expression in HaCaT cells by activating STAT3 and that Sh might inhibit the over-expression through interference of STAT3 signaling. Methods: In vitro culture of HaCaT cells treated with IFN-γ and measurement of K17 protein by enzyme linked immunosorbent assay. Results: The level of K17 protein (one kind of keratin protein) in the supernatant induced by IFN-γ was significantly reduced by Shikonin at various concentrations. Interference of STAT3 suppressed the effect of IFN-γ on K17 expression at both mRNA and protein levels. The over-expression of K17 in IFN-γ-induced HaCaT cells was significantly suppressed by 2 µg/L Shikonin. Interfering with STAT3 signaling with 2 µg/L Shikonin resulted in an intermediate level of IFN-γ-induced K17 protein in HaCaT cells. Conclusions: These data demonstrate that IFN-γ induces K17 protein over-expression of HaCaT cells by activating STAT3 and Shikonin may inhibit the over-expression partly through interference of STAT3.

Keywords: Shikonin, IFN-γ, K17, STAT3

Introduction
Psoriasis is an immunological skin disease characterized by epidermal hyperproliferation and chronic inflammation. Progress in the understanding of psoriasis has demonstrated it is a T cell mediated disease where the regulation of local and systemic cytokines plays an important role in its pathogenesis. Previous studies demonstrated that interleukin (IL)-17A is a key factor for the early onset of psoriasis and interferon (IFN)-γ is the central cytokine involved in the overall pathogenesis [1]. Cytoskeletal protein keratin 17 (K17) is overexpressed in psoriatic epidermis and considered to be a hallmark of psoriasis [2, 3]. The up-regulation of K17 protein in HaCaT cell after exposure to IFN-γ indicates a close relationship between IFN-γ and K17 [4]. This effect was further supported by another study showing that IFN-γ strongly induced the expression of the K17 gene promoter [5]. Notably, K17 is the only keratin induced by IFN-γ [6].

Lithospermum erythrorhizon (LE) belongs to Boraginaceae perennial. The roots of LE have anti-inflammatory, anti-tumor, hepatoprotection, immune regulation, contraception, sterilization, and other antivirus effects [7]. Shikonin (Sh) is one of the main components of LE. In a previous study, we found that 50 µmol/L of Sh inhibited the over-expression of IL-17-induced vascular endothelial cell growth factor (VEGF), IL-6 and IL-23 in the supernatant of cultured HaCaT cells [8]. In another study, we revealed that 5, 7.5, 10, and 12.5 µg/ml of Sh decreased the level of IL-17 in peripheral blood mononuclear cells (PBMCs) from psoriasis patients in a dose-dependent manner. A dose of 12.5 µg/ml Sh also reduced IL-17 and IL-6 levels induced by IL-23 in PBMCs from psoriasis patients [9].

Recent studies showed that IFN-γ, IL-6 and IL-22 stimulation of podoplanin (PDPN) expression is signal transducers and activators of transcription (STAT) 1- and STAT3-dependent [10]. Studies also showed that K17 expression in HaCaT cells was primarily regulated by STAT-dependent signaling pathways [11, 12]. A site within the promoter region of the K17 gene responds to IFN-γ via binding transcription factor STAT1 [11]. However, whether IFN-γ up-regul-
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lates K17 through STAT3 signaling pathways is unknown. Here, we hypothesized that IFN-γ induces K17 over-expression in HaCaT cells by activating STAT3 and that Sh might inhibit the over-expression through interference of STAT3 signaling. Our findings may contribute to the pathogenesis and treatment of psoriasis.

Methods

Cell culture and Sh treatment

HaCaT cells were cultured in DMEM (Dulbecco’s Modified Eagle Media) with 10% fetal bovine serum under a humidified atmosphere containing 5% CO₂ at 37°C. HaCaT cells at 1×10⁵ cells were plated into each well of a 24-well plate. After 24 hours, cells were co-cultured with IFN-γ (250 U/ml) (5) and Sh (0, 2, 5, 10, 12.5, 20 or 30 µg/L). Cyclosporine (10 µg/L) was used to replace Sh as a positive control because it inhibits HaCaT cell proliferation by induction apoptosis of HaCaT cells [13].

MTS for cell viability detection

The Cell Titer 96® Aqueous One Solution Reagent was used for MTS (Promega, America). The reagent was placed at room temperature for approximately 90 minutes until completely thawed, then 20 µl was dispensed into each well of a 96-well plate containing HaCaT cells and 100 µl culture medium. The plate was incubated at 37°C for 3 hours in a humidified atmosphere containing 5% CO₂. The absorbance at 490 nm was recorded using a 96-well plate reader.

K17 expression using enzyme linked immunosorbent assay

Figure 1. K17 expression in IFN-γ-induced and Shikonin-treated HaCaT cells. NC, negative control (no IFN-γ, no Shikonin); Sh, Shikonin; IFN, IFN-γ; PC, positive control (cyclosporine A10 µg/L + IFN-γ).

Figure 2. HaCaT cells activated by IFN-γ and treated with Shikonin. MTS assay measured cell activity from HaCaT cells. Sh, Shikonin; IFN, IFN-γ.
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Delaware State) at 37°C for 1 hour. Subsequently, the cells were incubated with 90 µl of TMB Substrate for 10 minutes at room temperature. The reaction was stopped with 50 µl of stop solution and the absorbance was measured at an optical reference wavelength of 450 nm with an ELISA reader.

Transfection of siRNA

Concentrations of 50-100 µM of AllStars negative control siRNA with green fluorescent protein (GFP) and STAT3-specific siRNAs were transfected into HaCaT cells using HiPerFect Transfection Reagent according to the manufacturer’s protocol (Qiagen, Hilden, Germany). STAT3 target sequence was: CAGGCTGGTAATT-TATATAAT.

RT-PCR for K17 mRNA detection

Total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) per the manufacturer’s instructions, and cDNA was synthesized with QuantiTect Rev., Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. This was used as the template for quantitative PCR. Subsequently, Real-time PCR was performed using the Quantifast SYBR green PCR kit (Qiagen Hilden, Germany). The reaction components were 0.5 ml of forward and reverse primers for K17 and GAPDH gene as an internal control. Primers sets were: 5'-ccacccagagaactgtggt-3', and 5'-ttctagacggcaggtcaggt-3' for the amplification of GAPDH and QuantiTect Primer Assays (Qiagen) were used for KRT17 (Hs_KRT17_1_SG, QT00001680), respectively. The cycling conditions were as follows: 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 15 seconds. All reactions were run in triplicate in at least three independent experiments.

Flow cytometry for K17 protein detection

Cells were resuspended at approximately 1-5×10⁶ cells/ml in ice cold PBS. The cells were sequentially incubated with primary and secondary antibodies (Abcam, USA). Appropriate isotype specific negative controls were used in all staining. Cell suspensions were immediately stored at 4°C in the dark.

Results

Effects of Sh on K17 expression in the supernatant of HaCaT cells induced by IFN-γ

The level of K17 protein in the supernatant from HaCaT cells treated with IFN-γ was high, but significantly decreased (P<0.01) when Shikonin was added at various concentrations (Figure 1). Thus, Sh inhibited the secretion of K17. For Sh, the optimal concentration that did not influence the survival rate of HaCaT cells was 2 µg/ml (Figure 2).

Expression of K17 and the effects of Sh on K17 expression in IFN-γ-induced HaCaT cells

Both RT-PCR (Figure 3) and flow cytometry (Figure 4) results showed significant differences between the IFN-γ only group and other four groups (IFN-γ+stat3-siRNA, IFN-γ+Sh, IFN-γ+Sh+stat3-siRNA and blank) (P<0.01). The K17 level was highest in the IFN-γ only group, and the lowest was in the IFN-γ+Sh group, indicating the high level of K17 induced by IFN-γ was significantly suppressed by Sh. The K17 expression of IFN-γ+stat3-siRNA group was lower than that of the IFN-γ only group, but higher than that of the negative control (P<0.01).
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suggesting STAT3 was in part involved in the inhibition of IFN-γ. When IFN-γ treated cells were incubated with Sh in the presence of STAT3-specific siRNA, K17 expression was higher than in the IFN-γ+Sh group and lower than the IFN-γ+stat3-siRNA group ($P<0.01$), indicating STAT3 was a target of Sh although other targets may exist.

**Discussion**

Many chemokines concomitant with an influx of T cells and inflammatory dendritic cells (DCs) can be up-regulated by IFN-γ in the skin. A study showed that IFN-γ-enhanced IL-23 and IL-1 production by DCs and subsequently induced Th17 cells [12]. IFN-γ has a synergetic effect with other psoriasis-related inflammatory cytokines such as IL-17, IL-22, and this synergy may be an important process in the pathogenesis of psoriasis [14, 15]. These facts support the notion that IFN-γ is a key pathogenic cytokine that can induce many features of the inflammatory cascade [16]. K17 is the only keratin reported to be induced by IFN-γ. It is highly expressed in psoriatic lesions, but is not expressed in the healthy epidermis. Our research confirmed that IFN-γ, via its induction of K17 expression, plays an important role in the regulation of keratinocytes.

STAT was first identified as a member of the cytosolic protein family during the study of interferon inducible gene transcription. STAT3 is one of the most important members of the family and mediates a variety of cytokine and growth factor signal transduction pathways to the nucleus to induce the transcription of target genes, thereby regulating cell function. It is also closely related to tumor occurrence, development and apoptosis [17, 18]. The abnormality of growth factor signaling plays an important role in the constitutive activation of STAT3, [19] the main contact between keratinized cells and immune cells [20]. STAT3 is involved in Th17 signaling and STAT3 was highly expressed in keratinocytes in psoriatic lesions [21]. Thus, STAT3 is considered to be closely associated with psoriasis.

Studies confirmed that IFN-γ and IL-17 upregulate K17 expression by activating STAT1 [10].
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and STAT1/3, respectively [21]. Another study showed that IFN-γ, IL-6, and IL-22 induced PDPN-expression of keratinocytes, and the upregulation was both STAT1- and STAT3-dependent [22]. In our experiment using STAT3 siRNA, K17 mRNA and protein expression induced by IFN-γ was significantly decreased; indicating IFN-γ induces K17 expression in HaCaT cells through STAT3.

The naphthoquinone pigment Sh is the most important pharmacologically active substance in the dried root of LE [23]. Sh can be categorized as a mitocan, a class of compounds that act by interfering with energy-generating mitochondrial processes, which in turn leads to reactive oxygen species accumulation, mitochondrial destabilization, and induction of apoptosis [24]. One study showed that Sh and its derivatives could inhibit VEGF-induced proliferation [25] and that Sh may be a promising agent for the treatment of psoriasis [26]. In the present study, we examined the inhibition role of Sh on IFN-γ-induced K17 expression and its mechanism of action. In the IFN-γ+Sh group, the expression of K17 was significantly decreased comparing to the IFN-γ only group, indicating Sh suppressed IFN-γ induced K17 expression. When IFN-γ and STAT3-siRNA were added to the cells, K17 expression decreased approximately 70%, indicating the IFNγ induction effect was partially dependent on STAT3. In the IFN-γ+Sh+siRNA group, K17 expression was further decreased compared to the IFN-γ+siRNA group, but higher than of the IFN-γ+Sh group. These results suggest STAT3 could be one of the targets of the therapeutic mechanism of Sh. Further investigation is warranted.

An improved understanding of the complex interplay between cytokines, their molecular signaling pathways in affected cells, and the resulting changes in these affected cells will allow a better understanding of the pathomechanisms involved in proliferative diseases including psoriasis. IFN-γ is an important cytokine in the autoimmune loop of K17, T cells and cytokines of psoriasis. The present study demonstrated that K17 expression is induced by IFN-γ stimulation in HaCaT cells via STAT3. Sh suppressed this pathway by inhibiting STAT3 signaling. Our findings may help to elucidate further the molecular and cellular mechanisms underlying the pathogenesis of psoriasis, and to provide new treatment strategies for this chronic disease.

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Disclosure of conflict of interest

None.

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