Original Article
Tripterygium wilfordii polyglycoside reduces the proliferation and inflammatory cytokines secretion of Hacat cells by regulating the balance of neutrophil elastase and trappin-2

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Abstract: Psoriasis is an immune-mediated inflammatory keratotic skin disorder. Although some endogenous stimuli have been clarified to involve into the pathophysiology of psoriatic, the targeted drugs for treating psoriasis are still limited. Tripterygium wilfordii polyglycoride (TWP) extracted from Tripterygium wilfordii has potent anti-inflammatory and immune suppression functions, which has been widely used to treat autoimmune and inflammation-related diseases. In this paper, two injury models in vitro including Neutrophil elastase (NE) and NE plus Tumor necrosis factor-alpha (TNF-α) treated Hacat cells were established to investigate the therapeutic effect of TWP on psoriasis. Hacat cells viabilities were detected by CCK-8 kits. In addition, the levels of interleukin-6 (IL-6), interleukin-8 (IL-8), NE and trappin-2 in the supernatant were assayed using ELISA kits; and the expression of intercellular adhesion molecule-1 (ICAM-1) was detected by western blotting. The results showed that the cell viabilities, NE and trappin-2 levels in Hacat cells were markedly increased after pretreatment with NE compared with the control group. However, TWP and trappin-2 could significantly down-regulated cell viabilities, NE and the NE/trappin-2 ratio. Furthermore, TNF-α and NE plus TNF-α could up-regulated the levels of IL-6 and IL-8 and the ICAM-1 protein expression, which were reversed by pretreatment with TWP or trappin-2. Thus, we concluded that TWP has inhibitory effects on proliferation and inflammatory cytokines secretion of Hacat cells by decreasing the ratio of NE and Trappin-2 level. These findings provide a new insight that will aid in elucidating the actions of TWP against psoriasis.

Keywords: Inflammatory cytokines, NE, psoriasis, trappin-2, TWP

Introduction
Psoriasis is a chronic inflammatory skin disease characterized by an aberrant keratinocyte proliferation and an altered immune activation [1, 2]. Recent researches have indicated that the incidence of psoriasis varies between 1% and 3% of the population worldwide and continues to rise [3, 4]. About 3-10% moderate psoriasis patients appear inflammation lesions. In addition, the comorbid conditions of psoriasis such as arthritis, non-alcoholic fatty liver sickness, osteoporosis, cardiovascular diseases and metabolic syndrome increase physical, psychological and financial burden [5-7].

Mechanistically, the direct trigger of psoriasis isn’t completely clarified, however the involvement of endogenous stimuli has been elucidated [8]. Neutrophil elastase (NE), one of the most crucial proteases released by neutrophils, plays a key role in the pathogenesis of psoriasis [9]. NE contributes to the removal of harmful bacteria and heterologous substances by phagocytic cells and the digestion of damaged tissue [10]. Furthermore, the over-expression of NE can strengthen adhesion between neutrophil and endothelial cells through promoting the expression of intercellular adhesion molecule-1 (ICAM-1) and the secretion of inflammatory factors [11, 12]. These pathological changes cause
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alterations in the hyperproliferation and differentiation of keratinocytes, dilatation of blood vessels, and infiltration of inflammatory cells into the epidermis and dermis [13]. Furthermore, the excessive release of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) have been detected in skin lesions and circulation of patients with psoriasis, and resulting in cascade reaction [14, 15]. Trappin-2 protein is an endogenous and specific NE inhibitors mainly secreted by keratinocytes and epithelial cells, only expressed in inflammatory skin disease [16]. Previous researches have shown that NE significantly promotes the proliferation and metabolism of HaCaT keratinocytes in vitro. In addition, Trappin-2 is involved in regulating the proliferation of HaCaT keratinocytes through suppressing the NE activity [16-18]. Therefore, the balance between NE and Trappin-2 plays an important role in proliferation and inflammation in psoriasis pathology.

Presently, early intervention in psoriasis patients has been proven to be beneficial to reduce systemic inflammation and clearance of skin lesions; however, the targeted drugs for psoriasis are limited because of the complex
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The main anti-psoriasis therapies include conventional systemic therapy (e.g., cyclosporine, methotrexate, acitretin and photochemotherapy) and biological agents (e.g., infliximab, etanercept, efalizumab and adalimumab). However, these therapies have many potential side effects including fever, fatigue, skin itching and other symptoms [21]. At present, only 25% of patients are satisfied with their treatment for psoriasis [22]. Besides these, single target spot and high price of these agents also restrict their developing and applying in the clinic. Thus, developing effective therapies for this disease is urgently needed.

Tripterygium wilfordii polycoride (TWP) is an aqueous-chloroform extract from traditional Chinese medicine Tripterygium, which has been widely used to treat autoimmune and inflammation-related diseases [23, 24]. Modern pharmacology researches indicated that TWP had potent anti-inflammatory and immune suppression functions [23]. In addition, TWP processes a therapeutic action for the psoriasis patients; however, the possible molecular mechanism of TWP against psoriasis remains unclear.

Thus, in this paper, we decided to further explore the actions of TWP against psoriasis and then to investigate whether balance of NE and Trappin-2 was involved in the underlying molecular mechanism.

Results

The effects of NE on cell proliferation and inflammatory factors of Hacat cells

As shown in Figure 1A, the viabilities of Hacat cells were markedly increased after 12, 24 and 48 h pretreatment with NE at the doses of 1 and 10 IU/L, but notably decreased after pretreatment with NE (50 and 100 IU/L) for 12, 24 and 48 h. These results indicated that NE could increase the Hacat cells viabilities at the low doses. However, high concentration of NE produced cytotoxic effects on Hacat cells. Furthermore, as shown in Figure 1B, 1C, NE at the concentrations of 0.1-100 IU/L slightly changed the IL-6 and IL-8 levels in Hacat cells with no significance (P>0.05). However, NE notably increased the Trappin-2 level in Hacat cells at the low doses but decreased Trappin-2 level at high doses (Figure 1D). After analyzing these data, we chose 24 h and 10 IU/L as the most appropriate pretreatment time and concentration dose for NE, respectively. In addition, as shown in Figure 2A-C, after pretreatment with 0.1-100 IU/L NE for 12, 24 and 48 h, the protein levels of ICAM-1 in Hacat cells were slightly changed with no significance (P>0.05).
The effects of recombinant protein trappin-2 on cell proliferation of Hacat cells

As shown in Figure 3, the viabilities of Hacat cells were slightly changed with no significance after treating with recombinant protein trappin-2 at the doses of 10-100 ng/ml for 12 h (P>0.05). However, pretreatment with trappin-2 at the doses of 25-100 ng/ml for 24 or 48 h could decrease notably the Hacat cells viabilities with a dose-dependent manner. After analyzing the data, we chose 24 h and 25 ng/ml as the most appropriate pretreatment time and concentration dose of trappin-2, respectively.

The cytotoxic effect of TWP on Hacat cells

As shown in Figure 4, compared with the control group (TWP = 0 μg/ml), TWP (1-100 μg/ml) slightly altered the Hacat cells viabilities with no significance, which indicated that TWP (P<100 μg/ml) has no significant cytotoxicity to Hacat cells.

The effects of TWP on NE-induced injury in Hacat cells

As shown in Figure 5, the viabilities of Hacat cells were markedly increased after 24 h pretreatment with NE (10 IU/L) compared with the control group (P<0.01). In addition, the Hacat cells viabilities weren’t significantly changed by TWP and trappin-2. However, compared to the NE group, 24 h pretreatment with TWP (50 μg/ml) or trappin-2 (25 ng/ml) could notably decrease the Hacat cells viabilities after treating with 10 IU/L NE for 24 h.

The effects of TWP on the balance of NE and trappin-2 in Hacat cells

As shown in Figure 6A, 6B, the NE and trappin-2 levels in Hacat cells were markedly increased after 24 h pretreatment with NE (10 IU/L) compared with the control group (P<0.01). In addition, the NE and trappin-2 levels weren’t significantly changed by TWP and trappin-2.
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Figure 7. A: The effects of TWP on inflammatory factors IL-6 and IL-8 levels in NE plus TNF-α co-treated Hacat cells. B: The effects of TWP on NE, trappin-2, and the NE/trappin-2 ratio in NE plus TNF-α co-treated Hacat cells. Data are presented as the mean ± SD (n = 8, "P<0.01 versus Control group; \#P<0.01 versus TNF-α group; \##P<0.01 versus NE plus TNF-α group).

The effects of TWP on NE plus TNF-α-induced injury in Hacat cells

As shown in Figure 7A, compared with control group, the inflammatory factors including IL-6 and IL-8 were significantly up-regulated in TNF-α (10 ng/ml) and NE (10 IU/L) plus TNF-α (10 ng/ml) groups. However, pretreatment with 50 µg/ml TWP or 25 ng/ml trappin-2, the IL-6 and IL-8 levels were decreased markedly compared with TNF-α group. In addition, TWP and trappin-2 could also notably down-regulated the IL-6 and IL-8 levels compared to NE plus TNF-α group. Furthermore, as shown in Figure 7B, NE and trappin-2 levels and NE/trappin-2 ratio were significantly increased after 24 h pretreatment with NE (10 IU/L) plus TNF-α (10 ng/ml) compared with control group. However, compared to the NE plus TNF-α group, 24 h pretreatment with TWP (50 µg/ml) or trappin-2 (25 ng/ml) could markedly increased the trappin-2 level and decreased the NE level and NE/trappin-2 ratio in Hacat cells.

Figure 8. The effects of TWP on inflammatory factor ICAM-1 level in NE plus TNF-α co-treated Hacat cells. Data are presented as the mean ± SD (n = 3, "P<0.01 versus Control group; \##P<0.01 versus NE plus TNF-α group).

However, compared to the NE group, 24 h pretreatment with TWP (50 µg/ml) or trappin-2 (25 ng/ml) could notably down-regulated the NE level and up-regulated the trappin-2 level in Hacat cells. Therefore, the ratio of NE and trappin-2 in Hacat cells was markedly increased compared with the control group, which was notably decreased by 50 µg/ml TWP or 25 ng/ml trappin-2 for 24 h pretreatment (Figure 6C).
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As shown in Figure 8, compared to the control group, the ICAM-1 protein expression in Hacat cells was significantly increased after 24 h pretreatment with NE (10 IU/L) plus TNF-α (10 ng/ml). However, ICAM-1 protein expression was notably down-regulated by TWP (50 ug/ml) or trappin-2 (25 ng/ml) for 24 h pretreatment compared to the NE plus TNF-α group.

Discussion

Psoriasis is a common inflammatory skin disease usually associated with red, scaly, and raised plaques caused by several comorbid conditions [25, 26]. Over the past decade, clinical and experimental studies have been confirmed that neutrophils not only produce various types of proteases with immunomodulation, but also synthesize and secrete highly active cytokines [27, 28]. Previous researches have shown that the balance between NE and Trappin-2 plays an important role in proliferation and inflammatory response [16]. NE stimulates the proliferation of keratinocytes through epidermal growth factor receptor (EGFR) signaling pathway, which can partly explains the accelerating proliferation of psoriatic skin lesions with neutrophil infiltration [29]. NE can also induce the expression of multiple cytokines and form a complex cytokine network, resulting in “Waterfall-like” cascade reaction [30]. Trappin-2 protein is a NE endogenous inhibitor mainly released from keratinocytes and epithelial cells, which is a marker of keratinocytes and tumor cells differentiation [31]. Trappin-2 can effectively inhibit the activity of NE through the degradation of NE, thereby indirectly inhibit cell proliferation [32].

In this paper, two damage models of HaCaT keratinocytes in vitro were used to study the effects and molecular mechanisms of TWP on psoriatic models. The present results showed that 24 h pretreatment with TWP (50 ug/ml) or trappin-2 (25 ng/ml) could notably decrease the Hacat cells viabilities compared to the NE group. In addition, compared to the NE group, 24 h pretreatment with TWP or trappin-2 could notably down-regulated the NE level and up-regulated the trappin-2 level in Hacat cells. Therefore, the ratio of NE and trappin-2 in Hacat cells was markedly increased compared with the control group, which was notably decreased by 50 ug/ml TWP or 25 ng/ml trappin-2 for 24 h pretreatment. Furthermore, compared to the NE plus TNF-α group, 24 h pretreatment with TWP (50 ug/ml) or trappin-2 (25 ng/ml) could markedly increased the trappin-2 level and decreased the NE level and NE/trappin-2 ratio in Hacat cells. These results proved that TWP has inhibitory effects on proliferation of Hacat cells by decreasing the ratio of NE and Trappin-2 level.

The excessive release of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), IL-6 and IL-8 have been detected in skin lesions and circulation of patients with psoriasis, and resulting in cascade reaction [33]. Keratinocytes produce anti-inflammatory and immunosuppressive factors induced by epidermal cell proliferation in psoriasis patients, and IL-6 is one of the most important mediator [34]. IL-6 also leads to the abnormal proliferation of keratinocytes through activation of MAPK pathways [35]. Previous studies also showed that the concentration of IL-8 in the skin plaques in psoriasis was greater than 100-fold compared with normal epidermis [36]. IL-8 leads to the formation and overgrowth of abnormal keratinocytes in psoriatic lesions, and promotes the production of vascular growth factor (VGF) to accelerate blood vessels formation and cell growth in the lesion [37].

The levels of IL-6, IL-8 in the supernatant of Hacat cells were assayed in this research. Compared with control group, the inflammatory factors including IL-6 and IL-8 were significantly up-regulated in TNF-α (10 ng/ml) and NE plus TNF-α groups. However, pretreatment with TWP (50 ug/ml) or trappin-2 (25 ng/ml), the IL-6 and IL-8 levels were decreased compared with TNF-α group. In addition, compared to NE plus TNF-α group, TWP and trappin-2 could notably down-regulated the IL-6 and IL-8 levels. These results indicated that TWP has inhibitory effects on TNF-α or co-treatment of NE with TNF-α induced IL-6 and IL-8 release in Hacat keratinocytes cells.

ICAM-1 is a member of immunoglobulin gene superfamily, mainly located in the most of vascular endothelial and epithelial cells [38]. Studies have shown that ICAM-1 play a role in the pathogenesis of inflammatory skin diseases through regulating lymphocyte moving and homing at the sites of inflammation [39]. In psoriasis and other skin inflammation process, various cytokines produced by keratinocytes...
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can induce expression of adhesion molecules in inflammatory and vascular endothelial cells [40]. This paper showed that ICAM-1 protein expression was notably down-regulated by TWP (50 μg/ml) or trappin-2 (25 ng/ml) for 24 h pretreatment compared to the NE plus TNF-α group. The results supported that TWP showed inhibitory effects on co-treatment of NE with TNF-α induced ICAM-1 expression in Hacat keratinocytes cells.

Materials and methods

Reagents and materials

Human IL-6, IL-8, NE, trappin-2 enzyme-linked immunosorbent assay (ELISA) Kit were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Fetal bovine serum (FBS, No. 10099-141) was from Gibco (California, USA). Dulbecco minimum essential medium (DMEM, No. SH30022.01B)-high glucose medium was from Hyclone Laboratories, Inc. (Massachusetts, USA). Penicillin-streptomycin, phosphate buffer saline (PBS) buffer and Cell Counting Kit-8 (CCK-8, No. CK04) were from Dojindo Molecular Technologies, Inc. Tissue Protein Extraction Kit (No. KGP2100) was from KEYGEN Biotech. Co., Ltd. (Nanjing, China). Bicinchnionic acid (BCA) Protein Assay Kit was from Beyotime Institute of Biotechnology (Shanghai, China). Anti-rabbit-ICAM-1 and Anti-GAPDH-conjugated affinipure IgG (H+L) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies were supplied by BOSTER (Wuhan, China).

Cells culture

The HaCaT cell line was obtained from the Institute of Biochemistry Cell Biology (Shanghai, China) and cultured in DMEM medium supplemented with 10% FBS, 100 U/ml Penicillin-streptomycin at 37°C in 5% CO₂ in a saturated humidified incubator (Thermo Fisher Scientific, Massachusetts, USA).

Cell Counting Kit-8 (CCK-8) assays

HaCaT cells were digested by 0.25% trypsin after washing with PBS, and cell density in the suspension was adjusted to 1 × 10⁵/ml. Then, 100 μL cell suspension was added into the 96-wells plate for culturing in a saturated humidified incubator. After adhering and growing for 24 h, HaCaT cells were treated according different tests. Then, the viabilities of HaCaT cells were detected according to the CCK-8 method. The absorbance of the samples was quantified at 450 nm using a spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

Inflammatory cytokine assays

Inflammatory cytokines in HaCaT cells were detected by using the enzyme-linked immunosorbent assay (ELISA) kits. Briefly, the HaCaT cells (1 × 10⁵ cells/ml) were seeded in 24-well culture plates, and then treated according different tests. The cells fixed by 4% paraformaldehyde were blocked with 2% bovine serum albumin (BSA). After exposure to antibodies for 2 h, followed by washing with cold PBS, the cells were incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h. The expression of IL-6, IL-8, NE and trappin-2 was quantified by the addition of peroxidase substrate solution and subsequent absorbance measurement at 490 nm using a microplate reader (Thermo, USA).

Western blotting assay

Total protein was extracted from the cultured cells with an appropriate cold lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and the protein concentration was determined by a BCA protein assay Kit. Samples were subjected to SDS-PAGE (10%-15%) and transferred onto a PVDF membrane (Millipore, USA). Membranes were blocked and incubated overnight at 4°C with the primary antibodies ICAM-1 or GAPDH. Membranes were before incubated at room temperature with an appropriate secondary antibody, and proteins were detected using an enhanced chemiluminescence (ECL) method. Protein bands were imaged using a ChemiDoc XRS system (Bio-Rad, USA). To eliminate the variation of protein expression, the data were adjusted to correspond internal reference expression (IOD value of target protein versus IOD of correspond internal reference).

Statistical analysis

The results were expressed as the mean ± SD. One-way repeated-measures ANOVA was used for the analysis of the differences between the
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groups. All statistical analyses were carried out by SPSS 18.0 software, P<0.05 or P<0.01 were considered to be statistically significant.

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Disclosure of conflict of interest

None.

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