Paeoniflorin suppresses inflammatory response in imiquimod-induced psoriasis-like mice and peripheral blood mononuclear cells (PBMCs) from psoriasis patients

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Paeoniflorin suppresses inflammatory response in imiquimod-induced psoriasis-like mice and peripheral blood mononuclear cells (PBMCs) from psoriasis patients

Tao Chen 1,2, Li-xin Fu 1, Li-wen Zhang 1, Bin Yin 1, Pei-mei Zhou 1, Na Cao 1, Yong-hong Lu 1

Tao Chen and Li-xin Fu contributed equally to this work

1 Department of Dermatovenereology, Chengdu Second People’s Hospital, Chengdu, 610017, Sichuan, China.

2 Department of Dermatovenereology, Chengdu Qingbaijiang Distinct People’s Hospital, Chengdu, 610300, Sichuan, China.

Correspondence to: Dr. Yong-hong Lu, Department of Dermatovenereology, Chengdu second people’s hospital, No.165, Caoshi street, Chengdu, 610041, Sichuan, China.

E-mail: CDSPhospital@163.com.
Psoriasis is one of the most common immune-mediated chronic inflammatory skin disorders, characterized by hyperproliferation of keratinocytes, dilation and growth of dermal capillary vasculature, and cellular infiltration of T cells, dendritic cells (DCs) and neutrophils. Paeoniflorin (PF), the principal component of total glucosides of paeony (TGP), displays anti-inflammatory and anti-oxidant properties in several animal models. In this study we investigated the anti-inflammatory effects and mechanisms of PF in imiquimod (IMQ)-induced psoriasis like mouse model. The effects of PF on inflammatory cytokines expression in peripheral blood mononuclear cells (PBMCs) from patients with psoriasis vulgaris were also observed. Our results indicated that PF effectively attenuated the clinical and histopathologic changes in IMQ-induced psoriasis like mouse model. Furthermore, PF reduced the infiltration of T cells, CD11c^+DCs and neutrophils in lesional skin. In addition, PF also significantly decreased the mRNA expression of inflammatory cytokines, such as IL-17, INF-γ, IL-6 and TNF-α in IMQ-induced psoriasis like mouse model and PBMCs from patients with psoriasis vulgaris. Hence, our data suggests that PF can inhibit leukocytes infiltration and decrease the expression of inflammatory cytokines such as IL-17, INF-γ, IL-6 and TNF-α. PF might be a candidate drug for the treatment of psoriasis.

Keywords: Paeoniflorin, imiquimod, psoriasis, PBMCs, inflammatory cytokines
Introduction

Psoriasis is an autoimmune-like chronic inflammatory skin disease affecting up to 3% of the population worldwide (Lowes et al. 2007). Histologically, psoriasis is characterized by hyperproliferation of keratinocytes, dilation and growth of dermal capillary vasculature, and cellular infiltration of T cells, dendritic cells (DCs) and neutrophils (Christensen et al. 2006; Hegyi et al. 2012; Smits et al. 2006; Sumida et al. 2014). Although the pathogenesis of psoriasis is not fully elucidated, it is widely accepted that cellular infiltration of T cells, DCs and neutrophils is important in the pathogenesis of psoriasis by provoking inflammation (Boehncke et al. 2015). Several studies also supported that abundant production of inflammatory cytokines, such as interleukin (IL)-17, interferon (IFN)-γ, IL-6 and tumor necrosis factor-α (TNF-α) plays a key role in the development of this disease (Clark et al. 2006; Lin et al. 2012; Lowes et al. 2008).

Total glucosides of paeony (TGP) is an effective constituent purified and extracted from the dried root of Paeonia. The *Paeonia lactiflora*, which is called ‘Shao Yao’ in Chinese, has been widely used in China as a therapeutic drug for pain, cramp, congestion and giddiness for more than 1000 years. Recently, TGP has been approved as a novel treatment of rheumatoid arthritis by State Food and Drug Administration (SFDA) of China. Paeoniflorin (PF) is the principal component of TGP, which has been reported with many pharmacological effects such as anti-oxidative, anti-inflammatory and hepatoprotective activities (Sun et al. 2009; Zhang et al. 2008). PF has been used for the treatment of systemic lupus erythematosus (SLE),
rheumatoid arthritis (RA), Sjögren's syndrome (SS) and other inflammatory and autoimmune diseases (Lin et al. 2012; Wang et al. 2014; Zhao et al. 2012).

In order to further extend the understanding of the pharmacological functions of PF and to provide experimental evidence for its potential clinical use in the treatment of psoriasis, the therapeutic effects and the mechanisms of PF in imiquimod (IMQ)-induced psoriasis like mouse model were investigated. The effects of PF on the expression of inflammatory cytokines, such as IL-17, INF-γ, IL-6 and TNF-α in lesional skin in IMQ-induced mice and peripheral blood mononuclear cells (PBMCs) from patients with psoriasis vulgaris were also investigated.

Materials and Methods

Ethics statement

Animal experiments were approved by the Institutional Animal Care and Use Committee of Chengdu Second People’s Hospital (Sichuan, China). Human psoriasis studies were approved by the Ethics Committee of Chengdu Second People’s Hospital (Sichuan, China). All patients were oriental, and gave written informed consent.

Mice

Six-week-old BALB/c male mice weighting 20-25g were selected for this experiment. The mice were maintained in specific pathogen-free (SPF) conditions.

Animal model

BALB/c mice were randomly divided into four groups (n =10 per group): Vaseline group, IMQ group, IMQ + PF 50mg/kg group and IMQ + PF 100mg/kg group. A
daily topical dose of 5% IMQ cream (IMQ group) or control Vaseline (Vaseline group) was applied to the shaved back skin of the mice for 7 consecutive days. PF (molecular weight 480.05, purity 97%) was supplied from Liwah Plant Extraction Technology Co., Ltd. (Ningbo, China). In this study, PF was dissolved in phosphate-buffered saline (PBS) and injected i.p. at different concentrations (IMQ + PF 50mg/kg group and IMQ + PF 100mg/kg group) 0.5 h after IMQ treatment in each day. After 7 days of IMQ administration, we observed the psoriasis symptoms on the backs of the mice and evaluated disease severity with a clinical score. The evaluations were performed by three blinded observers. The scoring system is based on the clinical Psoriasis Area and Severity Index, except for the affected skin area (Shibata et al. 2013). Erythema, Thickening and scaling were scored independently on scale from 0 to 4: 0 = none; 1 = slight; 2 = moderate; 3 = marked; and 4 = very marked. The cumulative score served as a measure of the severity of inflammation (scale 0–12).

**Immunohistochemistry**

After 7 days treatment, mice were euthanized. Tissue samples in different groups were obtained. The one part of them was frozen for real-time quantitative PCR, and the other part was used for HE stain and immunohistochemistry. The skin samples of mice were fixed with formalin and embedded in paraffin. Sections (~4 µm) were stained by hematoxylin and eosin and immunohistochemistry using antibody against CD3, CD11c and myeloperoxidase (MPO). Images were acquired using an Olympus BX60 microscope. Quantitative assessments of pathological changes, based on a
Baker score system, were performed in ten randomly fields (x100) of each HE-stained section to assess the severity of psoriasis (Baker et al. 1992). The epidermal proliferation was indicated by the epidermal thickness of back skins.

Cell culture

Twenty patients with psoriasis vulgaris (PV) from Chengdu Second People’s Hospital (Sichuan, China) were enrolled in this study. All patients with PV were diagnosed by typical histological finds that including psoriatic acanthosis and Munro’s microabscesses. PBMCs from these patients were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation and cultured in RPMI1640 medium containing 10% FCS, 100 mg/ml penicillin and streptomycin.

Cell viability assay

The effects of PF on cell viability were determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates (1×10⁴ cells/ well) and treated with PF (10 µM, 100 µM, 1 mM, 5 mM) for 12 h. Cells were washed with PBS twice. Then MTT solution (0.5 mg/ml) was added to each well and cells were incubated at 37°C for 4 h. After centrifuged, 150 µl of dimethylsulfoxide was added to per well for formazan solubilization. The optical densities (O.D.) at 570 nm were determined using a micro-plate reader.

Real-time quantitative PCR

The mRNA levels of IL-17, INF-γ, IL-6 and TNF-α were measured by real-time quantitative PCR. Cells from each patient were divided into two groups and seeded in 6-well plates (2×10⁵ cells/ well). Cells were treated with PF (1 mM) or PBS for 12 h.
Total RNA of back skin samples and PBMCs were extracted by Trizol reagent according to the instructions. RNAs samples with high purity (A260/A280 ratio within 1.8-2.1) were used in this study. The quality of RNAs was also assessed by electrophoresis on 1% denaturing Agarose Gel. Intact total RNA run on a denaturing gel had sharp 28S and 18S rRNA bands. The 28S rRNA band was approximately twice as intense as the 18S rRNA band. The cDNA was synthesized from the mRNA by a two-step reverse transcription using RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The GAPDH gene was used as the endogenous control. cDNA samples were amplified for the interested gene and GAPDH in a 20 µl reaction volume containing 10 µl SYBR Green Master Mix and 0.25 µM qPCR primer. The following primers were used: human IL-17 (P291322, Bioneer, Inc., Daejeon, Korea); human IL-6 (P211161, Bioneer, Inc., Daejeon, Korea); human TNF-α (P237423, Bioneer, Inc., Daejeon, Korea); human INF-γ (Catalog:HQP009467, GeneCopoeia, USA); mouse IL-17 (Catalog:MQP029457 GeneCopoeia, USA); mouse IL-6 (Catalog:MQP036632 GeneCopoeia, USA); mouse INF-γ (Catalog: MQP027401 GeneCopoeia, USA) and mouse TNF-α (Catalog:MQP031019 GeneCopoeia, USA). The qPCR conditions were 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s with a final extension at 72°C for 5 min. The mRNA levels of IL-17, INF-γ, IL-6 and TNF-α were expressed as relative mRNA levels compared with control and calculated after normalization to GAPDH. The $\Delta \Delta Ct$ method for relative mRNA expression was utilized.

**Statistical analysis**
All experiments were carried out in triplicate. Data was expressed as mean ± SD and compared by One-way analysis of variance using the Graphpad Prism 5 software. A $P$-value <0.05 was considered statistically significant.

Results

PF effectively attenuates psoriatic lesions in IMQ-induced psoriasis like mouse model

To test whether PF is beneficial in IMQ-induced psoriasis like mouse model, mice were treated with PF 50 mg/kg and 100 mg/kg. One week later, PF was well tolerated, and the mice did not lose weight and showed no signs of sickness. As shown in Fig. 1A, we observed the psoriatic lesions characterized by erythema, thickening and scaling in IMQ group. In contrast, PF significantly alleviated the clinical changes in the IMQ-treated mice. Moreover, we assessed disease severity by a clinical score system. We found that PF significantly reduced the semiquantitative clinical score at 7 days after IMQ challenge (Fig. 1B).

PF effectively alleviates the histopathologic changes in IMQ-induced psoriasis like mouse lesions

As presented in Fig. 2A, skin histology revealed epidermal thickening, focal parakeratosis, epidermal rete elongation and brisk cellular infiltration of mononuclear cells in the dermis in the IMQ group. PF effectively ameliorated the histological appearance and the infiltrated lymphocytes of the psoriatic lesions compared with the IMQ-control. Furthermore, a Baker score system was used to confirm the pathological score of HE-stained preparations. We indicated that the Baker score was
significantly increased in IMQ-induced mice relative to Vaseline control group. However, PF markedly reduced the Baker score for histopathologic changes at 7 day after IMQ induction (Fig.2B). In addition, we also found that PF effectively reduced the average epidermal thickness compared with the IMQ group (Fig.2C). These data shows that PF can effectively alleviate the histopathologic changes in IMQ-induced psoriasis like mouse lesions.

**PF reduces the infiltration of T cells, CD11c⁺DCs and neutrophils in IMQ-induced psoriasis like mouse lesions**

Cellular infiltration of neutrophils, DCs and T cells in lesions plays vital roles in the pathogenesis of psoriasis. Immunohistochemistry using antibody against CD3, MPO and CD11c showed the abundant infiltration of T cells, CD11c⁺DCs and neutrophils in dermis in the IMQ group. However, PF markedly reduced the cellular infiltration of T cells, CD11c⁺DCs and neutrophils in dermis when compared with IMQ group (Fig.3).

**PF significantly inhibits the expression of IL-17, INF-γ, IL-6 and TNF-α in IMQ-induced psoriasis like mouse model in vivo**

Inflammatory cytokines such as IL-17, INF-γ, IL-6 and TNF-α are critical in the pathogenesis of psoriasis. We assayed IL-17, INF-γ, IL-6 and TNF-α mRNA expression in the mice lesions by real-time quantitative PCR. As shown in Fig.4, IMQ treatment alone caused a markedly increase in the mRNA levels of IL-17, INF-γ, IL-6 and TNF-α in lesional skin of mice. However, treatment with PF significantly
attenuated these changes. Thus, we suggest that PF can decrease the expression of IL-17, INF-\(\gamma\), IL-6 and TNF-\(\alpha\) in IMQ-induced psoriasis like mouse model in vivo.

**Effects of PF on the percentage of cell viability of PBMCs from psoriasis patients in vitro**

Cells were treated with PF at concentrations ranging from 10 \(\mu\)M to 5 mM for 12 h. MTT assay was performed to determine the cytotoxicity. As presented in Fig.S1, no significant detriment to cell viability was observed when cells were treated with PF < 1 mM for 12 h. However, after exposure to 5 mM PF for 12 h, cell viability decreased to (83.07\(\pm\)15.73) \% of the control group. Thus, 1 mM PF was used as the maximum dose throughout subsequent experiments to exclude toxicity.

**PF inhibits inflammatory cytokines such as IL-17, INF-\(\gamma\), IL-6 and TNF-\(\alpha\) expression at mRNA levels in PBMCs from psoriasis patients in vitro**

To further clarify the anti-inflammatory effects of PF, the mRNA expression of IL-17, INF-\(\gamma\), IL-6 and TNF-\(\alpha\) in PF treated PBMCs from psoriasis patients were investigated by real-time quantitative PCR. Compared with control group, the mRNA levels of IL-17, INF-\(\gamma\), IL-6 and TNF-\(\alpha\) were significantly reduced in the PF(1 mM)-treated group (Fig.5).

**Discussion**

Chemical compounds derived from plants used in traditional Chinese medicine to cure disease are important sources for the screening of new, active pharmaceutical molecules. Recently, it has been reported that TGP (which contains more than 90\% of PF) is a safe and effective medicine in the treatment of various inflammatory and
autoimmune disease, such as SLE, RA, SS and psoriasis (Chen et al. 2011; Zhang et al. 2008; Zhao et al. 2012). Nevertheless, the molecular mechanisms underlying the therapeutic effects of PF are not yet fully understood. Our previous study indicated that PF (50 mg/kg) can reduce vascular damage and perivascular leukocyte infiltrates in a mouse model of cutaneous vasculitis (Chen et al. 2013). In this study, we explored the potential therapeutic effects and mechanisms of PF in IMQ-induced psoriasis-like mouse model. We found that PF can effectively ameliorate the clinical and histopathologic changes in IMQ-induced psoriasis-like mouse model.

Psoriasis is a chronic inflammatory skin disease (Armstrong et al. 2013; Boehncke et al. 2015; Griffiths and Barker 2007). The common pathophysiological feature in psoriasis is epidermal hyperproliferation and leukocyte infiltrates (Ganguly et al. 2013). In this study, we also demonstrated that PF can reduce epidermal thickness and inhibit the cellular infiltration of T cells, CD11c+DCs and neutrophils in lesional skin in IMQ-induced mice. Data suggest that PF may be a potent anti-inflammatory agent and useful in treatment of psoriasis inflammation in IMQ-induced psoriasis-like mouse model.

An increasing numbers of studies have demonstrated that Th1/Th17 cells are important in the pathogenesis of psoriasis (Clark and Kupper 2006; Lin et al. 2011; Lowes et al. 2008). Psoriatic lesions also can produce many mediators and inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-17, IL-23 and IFN-γ, which contribute to the development of inflammatory response in psoriasis (Ganguly et al. 2013). Recently, the importance role of DCs in the immunopathogenesis of psoriasis
has been recognized (Lowes et al. 2005; Nestle et al. 2005). DCs can active T cells by presenting antigen molecules to them. The interactions with DCs and T cells also drive T cell priming and effector differentiation (Yan et al. 2014).

Sun et al. reported that PF attenuated psoriatic skin lesions and inflammation by reducing the number of neutrophils, dermal macrophages and Th1/Th17-associated cytokine production in the skin of IMQ-challenged mice (Sun et al. 2015). They also found that PF relieves epidermal hyperplasia by reducing the expression of TNF-α, IL-17, IL-21 and IL-22 (Sun et al. 2015). Lin et al. indicated that TGP ameliorated inflammation and immune response in RA by decreased production of IL-12 and IL-6, Th1- and Th17-polarizing cytokines which led to damaged Th1 and Th17 differentiation by attenuated phosphorylation of STAT1 and STAT3 (Lin et al. 2012). Similarly, in this study, we also indicated that PF effectively suppressed the number of T cells, inhibited the expression of IL-17, INF-γ, IL-6 and TNF-α in lesional skin in IMQ-induced mice. In addition, we also found that the expression of IL-4 and IL-10 had not been affected in preliminary study (data not shown). Hence, we suggest that the mechanism of PF in IMQ-induced psoriasis-like mice might be associated with the selectivity damage of abnormal Th1 and Th17 cells, and the reduction of inflammatory cytokines, such as IL-17, INF-γ, IL-6 and TNF-α. To further confirm these effects of PF in human diseases, an in vitro experiment was performed. We found that PF can block the mRNA expression of IL-17, INF-γ, IL-6 and TNF-α in PBMCs from psoriasis patients. This observation is unlikely to be due to the direct cell toxicity of PF on human PBMCs, because in a supplementary study about 95 %
of Cell viability was measured when incubated with PF (1 mM) for 12 h by MTT assay. We suggested that PF (1 mM) dose not affect cell viability of PBMCs from healthy control (Fig.S1). Moreover, the mRNA expression of IL-4 and IL-10 in PBMCs from psoriasis patients could not be inhibited by PF (data not shown).

In summary, our results provide new evidences for the anti-inflammatory effects of PF in IMQ-induced psoriasis like mice and cultured PBMCs from psoriasis patients. As showed above, we indicated that PF mainly effectively attenuated the clinical and histopathologic changes in IMQ-induced psoriasis like mouse model. The effects of PF might be associated with the inhibition of leukocytes infiltration and the decrease of inflammatory cytokines such as IL-17, INF-γ, IL-6 and TNF-α in IMQ-induced psoriasis like mouse model and PBMCs from patients with psoriasis vulgaris. These results provide an effective foundation and new insight into the immune pathogenesis of PF for the treatment in psoriasis. Further studies are now underway to address the potential additional effects of PF in psoriasis.

Acknowledgments

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Figure legends

Fig. 1

PF alleviates the psoriasis-like phenotype in IMQ-induced psoriasis like mouse model.

(A) The macroscopic characteristics of the back skins in the mice treated as described.

(B) A composite clinical score (CS) was based on the clinical Psoriasis Area and Severity Index for assess disease severity. Statistical analysis was conducted using One-way analysis. Values are expressed as means ± SD, n=10 per group. ##P<0.01, ###P<0.001, compared with Vaseline control group; ###P<0.001, compared with IMQ group.

Fig. 2

Histological appearance of back skins in the mice treated as described. (A) HE staining of tissue sections. Scale bars, 400 µm. (B) Quantitative assessments of pathological score (means ± SD, n=10 per group), based on a Baker score system, were performed in ten randomly fields (x100) of HE-stained sections to evaluate the severity of psoriasis. (C) Average epidermal thickness of all groups (means ± SD, n=10 per group). One-way analysis was used for statistical analysis. P<0.05 was considered to indicate a statistically significant result. ##P<0.01, compared with Vaseline control group; ###P<0.001, compared with IMQ group.

Fig. 3

Immunohistochemistry using antibody against CD3, CD11c and myeloperoxidase (MPO). Mice were treated as described above (ten mice per group). Skin sections were stained with antibodies against CD3, CD11c and MPO. These results were representative of multiple experiments and microscopic fields. Scale bars, 200 µm.
Fig. 4

The levels of IL-17, INF-γ, IL-6 and TNF-α mRNA from back skins in mice (n = 10 per group). GAPDH was used as internal reference gene. One-way analysis was performed and found that the levels of IL-17, INF-γ, IL-6 and TNF-α mRNA from back skins in mice with PF were significantly lower than IMQ group. *P<0.05, **P<0.01, compared with IMQ group; #P<0.05, ##P<0.01, ###P<0.001, compared with Vaseline control group.

Fig. 5

The mRNA expression of IL-17, INF-γ, IL-6 and TNF-α in PF treated PBMCs of psoriasis patients (n = 10 per group). The GAPDH gene was used as the endogenous control. Statistical analysis was conducted using t-test. P<0.05 was considered to indicate a statistically significant result. *P<0.05, **P<0.01.
Figure 1. PF alleviates the psoriasis-like phenotype in IMQ-induced psoriasis like mouse model. (A) The macroscopic characteristics of the back skins in the mice treated as described. (B) A composite clinical score (CS) was based on the clinical Psoriasis Area and Severity Index for assess disease severity. Statistical analysis was conducted using One-way analysis. Values are expressed as means ± SD, n=10 per group. **P<0.01, ###P<0.001, compared with Vaseline control group; ***P<0.001, compared with IMQ group.
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49x31mm (300 x 300 DPI)
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57x41mm (600 x 600 DPI)
Figure 5. The mRNA expression of IL-17, INF-γ, IL-6 and TNF-α in PF treated PBMCs of psoriasis patients (n = 10 per group). The GAPDH gene was used as the endogenous control. Statistical analysis was conducted using t-test. P<0.05 was considered to indicate a statistically significant result. *P<0.05, **P<0.01.