Original Article

Paeoniflorin strongly reduces the skin inflammation in psoriasis mice by inhibition of VEGF

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Abstract: Psoriasis, an immune-mediated inflammatory skin disease, is associated with increased dermal vascularization. Vascular remodeling is a hallmark of many chronic inflammatory disorders and vascular endothelial growth factor (VEGF) is key regulator in pathogenesis of psoriasis. Paeoniflorin (PF) is a monoterpene glucoside that widely used in Traditional Chinese Medicine. In this study, we attempt to elucidate therapeutic effect of PF on psoriasis, by its inhibitive role on VEGF production. Forty female mice were subjected to this experiment and randomly divided into four different groups. Psoriasis was induced by giving imiquimod (IMQ) cream for a week and treated with PF and/or PF-VEGF recombinant treatment. The expression levels of VEGF and its isotopes VEGF1 and VEGF2 were measured at serum level by ELISA, mRNA level by quantitative RT-PCR and protein level by western blot analysis. Epidermal abnormality of psoriatic skin lesions was determined by measuring keratin 6, keratin 10, loricrin and Ki67 at mRNA and protein level with RT-PCR and western blot analysis. Pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and innate immune mediators IL-22 and IL-12 p40 were detected by quantitative RT-PCR and western blot. Typical psoriatic histological features were observed in our histopathological analysis and epidermal abnormality of psoriatic skin lesions improved with PF treatment leading to a reduction in the number of blood vessels, this improvement was attenuated in PF-VEGF-treated mice. Expressions of VEGF, VEGF1 and VEGF2 at serum, mRNA and protein levels were significantly reduced in PF-treated mice compared with normal control mice, and this reduction was reversed by PF-VEGF treatment. The related markers keratin 6, keratin 10, loricrin, Ki67, TNF-α, IL-1β, IL-6, IL-22 and IL-12 p40 were decreased in PF-treated mice indicating the improvement in epidermal keratinocytes and inflammatory cells in psoriatic skin lesions. In contrast, these improvements were eliminated in PF-VEGF treated mice. In conclusion, our results suggest that systemic treatment of PF could induce inhibition of VEGF production and provide a proof for anti-inflammatory and anti-angiogenic effect of PF against psoriasis.

Keywords: Psoriasis, paeoniflorin, vascular endothelial growth factor (VEGF)

Introduction

Psoriasis is T-cell-mediated chronic inflammatory disease with overall prevalence of 2%-3% in the worldwide population [1]. Psoriasis has been characterized by epidermal hyperplasia with dysregulation of keratinocyte and inflammatory cell infiltration as well as increased vascularization [2, 3]. In psoriasis, angiogenesis has been featured with enlarged, tortuous, hyperpermeable blood vessels and enlarged lymphatic vessels [4, 5]. Vascular endothelial growth factor (VEGF) plays crucial role in regulating the neovascularization during the pathological processes and VEGF-induced angiogenesis is known to be one of the pathogenetic mechanisms in psoriasis [6]. Several studies have presented that high serum levels of VEGF were detected in psoriatic patients with significant correlation with psoriatic severity [7, 8]. Moreover, psoriasis has also reported to be associated with pronounced vascular permeability abnormality and excess VEGF production [2]. Thereby, great amount of efforts made on psoriasis therapy by regulating VEGF [9, 10]. However, due to its complex etiology and viciousness of chemical therapy, currently applied drugs for psoriasis is not fully satisfactory in clinical treatment.

Paeoniflorin (PF) is the principal component of total glucosides of paeony (TGP). With its anti-
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inflammatory, immune-regulatory and hepatoprotective functions, PF has been widely used in Traditional Chinese Medicine [11, 12]. Utilization of PF in treatment has been well documented in diverse disease, such as inflammation in Parkinson’s disease [13], chronic renal failure [14], ischemia and neurodegeneration [15], rheumatoid arthritis [16]. Although several studies attempt to define the anti-inflammatory effect of PF in psoriasis [17], potential role of PF on increased vascularization in pathogenesis of psoriasis is not fully elucidated.

A recent study reported that neutrophils release pro-inflammatory factors and, interestingly, IL-17 as well in psoriatic skin lesion [18]. Cutaneous macrophages also reported to produce diverse mediators and cytokines, such as TNF-α, IL-1β and IL-6, which play important role in the development of skin inflammation in psoriasis [19]. It has been demonstrated that T-cell dysregulation considered to be another pathogenetic explanation for psoriasis that usually present as increased level of Th1/Th17 in psoriatic lesions [20]. Although bioactive drugs used as TNF and IL-12/23 inhibitors (etanercept, adalimumab, infliximab and ustekinumab) widely applied in psoriasis treatment with relatively high efficacy, high rates of serious adverse events and propensity for viral infection have been observed in previous reports [21, 22].

In this study, we attempt to investigate the anti-inflammatory effects of PF in psoriasis-like phenotype rat model via regulation of VEGF in psoriasis skin lesions and to define the molecular pathogenesis by measuring the inflammatory related factors in different biological processes.

Materials and methods

Animals

Forty female 8-11w Balb/c mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science (Shanghai, China). The mice were maintained under pathogen-free conditions. All of the experiments were performed according to the Animal Care and Use Committee guidelines.

Grouping, induction of psoriasis and treatment

All the mice were divided into four different groups, with ten mice each (n = 10). Back hair of mice were completely shaved and wiped up with Na2S. The grouping of mice was randomized as following criteria: 1) for Normal Control (Normal) group, the mice received Vaseline on the shaved back on daily basis for consecutive 7 days with no other treatment; 2) for Negative Control (NC) group, the mice received imiquimod (IMQ) cream (Mingxi Pharma, Sichuan, China) for a week and intraperitoneal injection of normal saline for consecutive 10 days; 3) for paeoniflorin treatment (PF) group, the imiquimod was given to mice for a week prior to intraperitoneal injection of PF (Liwah Plant Extrat Technology, Ningbo, China) at the dose of 200 mg/kg/day, with purity of 96%, for consecutive 10 days; 4) for PF-VEGF recombinant treatment (PF-VEGF) group, imiquimod was given as described previously. After 6 hours of intraperitoneal injection of PF, subcutaneous injection of VEGF protein was given on the back of mice and this procedure was conducted for consecutive 10 days. The appearance of the psoriasis-like phenotype was determined by macroscopically visible scaly areas on the back of mice. Mice were euthanized at 18th day of treatment prior to preparing tissue and blood samples.

H&E staining

The skin of the euthanized mice was removed and fixed in 4% paraformaldehyde solution to be paraffin embedded. Tissue slices were cut with thickness of 4 μm from paraffin sections and stained with H&E and examined by light microscopy for histological analysis.

Quantitative real-time PCR

Total mRNA was extracted from the back skin tissue samples obtained from euthanized mice, using TRizol (Invitrogen, US) according to the manufacturer’s instructions. RNA (1 μg) was used for reverse transcription reaction with cDNA synthesis kit (Promega, US). The sequences of RT-PCR primers used for amplification of GADPH, VEGFA, VEGFR1, VEGFR2, Keratin 6A, Keratin 10, Loricrin, Ki67, TNF-α, IL-1β, IL-6, IL-22, IL-12 p40 are shown in Table 1. The reactions were setup in 25 μL of total volume and 0.1 μL of HotstartTaq DNA polymerase (GIAGEN, Germany), with 2 μL of template. The PCR cycle conditions was 95°C for 3 mins, 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 5 min for final extension. Bio-Rad iQ-5 (Bio-Rad, US) was performed for the amplifica-
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Table 1. Primer sequence of candidate genes (5'→3')

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>CGGAAGTCACCGATGGGGTCTGAT</td>
<td>AGCCCTCTCCATGCTGTTGGAAGAC</td>
</tr>
<tr>
<td>VEGFA</td>
<td>CACTGGACCTGGGTTACCTGAC</td>
<td>CCTACGCTCGGCTTGCTCAC</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>GAAAACACAGAAAGGACAG</td>
<td>CTTTATGCCCCAGAAGATCG</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>ATGGCATCGTTGCTACATCAC</td>
<td>TGCCCTACAGAAGAGATCG</td>
</tr>
<tr>
<td>Keratin 6A</td>
<td>GTGGGCCCTGCTCCCTTACAC</td>
<td>TCTGAGCAGGGGAGTCTG</td>
</tr>
<tr>
<td>Keratin 10</td>
<td>ACGAGAAGCATGGCAACTCA</td>
<td>GCCAGCCCTTCCCTGAGACT</td>
</tr>
<tr>
<td>Loricrin</td>
<td>TCACATCAGCATCACCTCCTTC</td>
<td>GGGAGGTGGGTGCTTCTTG</td>
</tr>
<tr>
<td>Ki67</td>
<td>CAGACCATCCTGGGCTGACT</td>
<td>AGGCAGCTGGATACGATG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GGCAGGCTCTATTGGAGTCTGCTG</td>
<td>ACATTCGAGGGCTCCAGTGAATCG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGCCACCTTTGAGGTGATG</td>
<td>AGGGTCCAGGGAGAAGAC</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCATCGAGGCTTCTGCTTGG</td>
<td>TTTCAATGGGAGTCTACATC</td>
</tr>
<tr>
<td>IL-22</td>
<td>AGGAGGTGGGCTTCCACCTACC</td>
<td>TGGATGTTCTGGTCACC</td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>GCTGGGTGTCCATGGCTTGGT</td>
<td>GGTAGGCTTCTCCAGAGAC</td>
</tr>
</tbody>
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tion and analysis of the PCR productions. GAPDH was used as internal reference. The 2^-ΔΔCT method was used for analyzing the relative mRNA expression.

Western blot analysis

A total of 30 mg of protein samples extracted from back skin tissues of mice using lysis buffer. BCA protein assay kit was used to measure the quality and concentration of proteins in supernatants. The cell lysates were separated on 10% SDS-PAGE gel and transferred to PVDF membranes. The membrane was blocked with 3% BSA and incubated with primary antibodies against VEGF, VEGFR1, VEGFR2, Keratin 6, Keratin 10, loricrin and Ki67 (Abcam, US) at dilution rate of 1:1000 with TBST. The membranes were then washed five times with TBST. The membranes were incubated with secondary antibodies (Beyotime Biotechnology, Shanghai, China) for 40 min at room temperature and washed as described above. The target proteins were examined using the ECL system (Millipore) and visualized using autoradiography film. Finally, the films were scanned and bands quantified using the Quantity one analysis software.

Enzyme-linked immunosorbent assay (ELISA)

The VEGF level in peripheral blood was measured by commercial ELISA assay kit (eBioscience, USA). The procedure was conducted as following the manufacturer’s instructions. After 15-30 min of antibody-antigen complex reaction, optical density (OD) was determined using a benchmark multiplate reader at 415 nm (Bio-Rad, Hercules, CA, USA).

Statistical analysis

For statistical evaluation SPSS 19.0 software was applied. For assessment of relative values, one-sample Student’s -tests was used by assessment of statistical significance of differences. One-way ANOVA analysis of variance with Tukey test was used to evaluate the differences between absolute means, when data were of normal distribution and homogeneity of variances was confirmed. Differences were considered statistically significant if the probability value was below 0.05 (P < 0.05).

Results

The relief of psoriasis-like phenotype in mice by paeoniflorin (PF) was antagonized by recombinant VEGF

First symptoms of a psoriatic phenotype were determined macroscopically observation of scaly areas on back skin of mice. The negative control and the mice treated with PF-VEGF recombinant injection showed predominant scaly skin lesion. In contrast, the mice treated with PF showed an almost complete reduction of scaliness, skin inflammation. The normal control mice did not show any epidermal abnormality (Figure 1A).

Histological analysis was performed to characterize the reduction of psoriasis-like skin inflammation in the mice treated with PF and scaly skin lesions on the back skin of mice injected with PF-VEGF recombinant agent. H&E staining exhibited the typical pathological features of the psoriasis-like phenotype in the negative control and PF-VEGF treated mice. In these mice, histopathological signs of psoriasis were observed, such as thickened epidermis and stratum corneum, retention of nuclei in the stratum corneum, downward thickenings of epidermis-known as rete pegs- and microabscesses. In
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Contrast, no pathological abnormality was observed in normal control mice and noticeable reduction of psoriatic histological features was shown in mice treated with PF-VEGF recombinant agent (Figure 1B). In accordance with histological observation, number of blood vessels in each group of mice was counted. Blood vessel numbers were significantly higher in negative control mice compared to normal mice ($P < 0.05$) and significant reduction was shown in PF-treated mice compared to negative control ($P < 0.05$) (Figure 1C). However, PF-VEGF treatment group showed no significant reduction in number of blood vessels compared to negative control group. These results indicated that VEGF affected the anti-angiogenesis effect of PF, and therefore it may at least partly participate in the PF-mediated psoriasis inhibition.

PF exerted inhibitive effect on VEGF secretion and the expression of its receptors, but recombinant VEGF abolished it

To investigate the effect of PF on expression of VEGF in serum at transcriptional and translational level, we examined the expression of VEGF, VEGFR1 and VEGFR2. ELISA analysis exhibited that peripheral blood serum in normal group and PF group contained significantly less VEGF than negative control mice and this scarcity in serum was restored after recombinant treatment of PF-VEGF ($P < 0.05$) (Figure 2A). In accordance with our western blot analysis, it was shown that protein expressions of VEGF, VEGFR1 and VEGFR2 were higher in negative control mice and significantly reduced with PF treatment ($P < 0.01$), and this reduction was significantly altered in mice treated with PF-VEGF ($P < 0.05$) (Figure 2B and 2C). Quantitative RT-PCR analysis was performed to examine the change at mRNA level of VEGF, VEGFR1 and VEGFR2 between different groups and we found that alteration of these genes at mRNA level had same trend as protein expression. The mRNA expression of these three genes was higher in negative control mice and significantly decreased with PF treatment ($P < 0.05$), and mRNA expression level increased again by PF-VEGF administration ($P < 0.05$) (Figure 2D).

PF-induced inhibition of hyperplasia and keratinization of epidermis was eliminated by recombinant VEGF

Histological analysis demonstrated that epidermal differentiation in psoriasis-like phenotype

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**Figure 1.** Macroscopic observation and H&E staining of skin lesions of differently treated mice. A. Macroscopic observation of scaly areas on back skin of mice; B. Histopathological signs of psoriasis in mice treated differently; C. Quantification of number of blood vessels in accordance with the histological observation ($^* P < 0.05$ compared with normal group; $^\# P < 0.05$ compared to negative control).
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mice was normalized with PF treatment and this effect was vanished by recombinant VEGF treatment. Western blot and quantitative RT-PCR analysis were performed to confirm this result at molecular level, examining the expression level of epidermal hyperplasia marker keratin 6, epidermal differentiation-related protein keratin 10, epidermal keratinization marker loricrin and proliferating cell nuclear antigen Ki67. These markers are known to be overexpressed in psoriasis lesions. Our western blot analysis showed that protein expression levels of keratin 6, keratin 10, loricrin and Ki67 were significantly decreased in mice treated with PF ($P < 0.05$) compared with psoriasis-like phenotype mice, and this reduction was significantly increased ($P < 0.05$) by administration of PF-VEGF recombinant agent (Figure 3A and 3B). This trend was further confirmed with the measurement of mRNA expressions of keratin 6, keratin 10, loricrin and Ki67 genes by quantitative RT-PCR. We found that mRNA expression level of these genes significantly decreased ($P < 0.05$) after PF treatment compared to psoriasis mice with no treatment (negative control), and this fall in mRNA expression was reverted to the increased level by PF-VEGF recombinant administration with statistical significance ($P < 0.05$) compared to PF treatment group (Figure 3C).

**Inhibition of skin inflammation by PF was neutralized by recombinant VEGF**

To better define the inhibitive effect of PF in pathogenesis of psoriasis by regulating the VEGF, we examined the protein and mRNA expression levels of TNF-α, L-1β, IL-6, L-22 and IL-12 p40, which are known as markers of inflammatory infiltrate and innate immune mediators that overexpressed in psoriasis. Western blot analysis revealed that protein expression levels of these inflammatory factors were significantly reduced ($P < 0.05$) in PF-treated mice compared with psoriasis-like phenotype mice and these decreased levels of proteins were remarkably increased ($P < 0.05$) after PF-VEGF administration (Figure 4A and 4B). Our quantitative RT-PCR analysis demonstrated the same
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**Figure 3.** Protein and mRNA levels of epidermal hyperplasia marker keratin 6, epidermal differentiation-related protein keratin 10, epidermal keratinization marker loricrin and proliferating cell nuclear antigen Ki67. A, B. Keratin 6, keratin 10, loricrin and Ki67 in the skin lesions measured with western blotting. C. mRNA levels of keratin 6 and loricrin in the skin lesions measured with qRT-PCR. **P < 0.01, *P < 0.05, compared with Normal control; ## P < 0.01, # P < 0.05, compared with NC (negative control); ※※ P < 0.01, ※ P < 0.05, compared with PF treatment group.

Discussion

Psoriasis is chronic autoimmune disease which has been reported to be characterized by poor differentiation and hyper-proliferation of epidermal keratinocytes [1]. PF has been reported to have anti-inflammatory, anti-allergic and immunoregulatory effects [11]. Our study, employing a unique therapeutic intervention model, reveals that systemic administration of PF could effectively alleviate the psoriasis-like phenotype skin inflammation and this effect is eliminated by PF-VEGF recombinant treatment. Histopathological characteristics of psoriasis-like symptoms were observed in the skin lesions of mice in experimental groups, such as epidermal hyperplasia, dermal and epidermal inflammatory infiltration, dilation and growth of dermal vessel, and elevated levels of related inflammatory cytokines, were markedly ameliorated following treatment with PF. In contrast, these improvements were exacerbated by administration of PF-VEGF recombinant agent.

To provide further confirmation for our histological observation, molecular parameters that related to epidermal proliferation and differentiation markers, such as keratin 6, keratin 10, loricrin and Ki67 were detected at mRNA and protein level in skin lesions. Our results showed that these markers were up-regulated in psoriatic phenotype mice and normalized with PF treatment in both mRNA and protein level. However, PF-VEGF recombinant treatment altered this normalization to the level of psoriatic skin lesions. Using Zenon labeling technique, van Duijnhoven MW et al. demonstrated that expression levels of Ki-7, keratin 6 and keratin 10 were detected at mRNA and protein level in skin lesions. Our results showed that these markers were up-regulated in psoriatic phenotype mice and normalized with PF treatment in both mRNA and protein level. Although, keratin 6 and keratin 10 are both expressed suprabasal layer of skin, their ex-
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Figure 4. Inhibition of skin inflammation by PF was neutralized by recombinant VEGF. A, B. Protein expressions of TNF-α, IL-1β, IL-6, IL-22 and IL-12 p40 were examined with western blotting analysis. C. mRNA expressions of these markers were detected with qRT-PCR analysis. **P < 0.01, *P < 0.05, compared with Normal control; ##P < 0.01, #P < 0.05, compared with NC (negative control); ※※P < 0.01, ※P < 0.05, compared with PF treatment group.

Expression patterns (co-or single-expression) in different stage of psoriatic lesions had been controversial till 2000. The more proportion of keratin 6-expressing cells presented in inner margin, while keratin 10-expressing cells are exist in the outer margin and K6/K10-co expressing cells were found in the inner margin of the lesions [24]. Thereby, we did not conduct further experiment for localization of these markers in different skin lesions with psoriasis, since this is not the scope of the present study. A study reported that treatment of NHKS with S100A7 significantly reduced the expression levels of keratin 10 and loricrin in psoriasis, and yet increased the expression of keratin 6, keeping certain discrepancy from our results [25].

There are three receptors for VEGF: VEGFR-1, VEGFR-2 [26] and VEGFR-3 [27]; all are tyrosine kinase receptors (RTKs), with high affinity. The potential role of VEGF in psoriatic pathogenesis is strongly correlated with the polymorphisms of VEGF gene [28, 29] and the overexpression of VEGF-receptors-1/2/3 in the psoriatic patients [30]. VEGFR-1 plays important role in normal blood vessel development, recruiting the hematopoietic precursors and leading migration of monocytes, while the VEGFR-2 determines the migration and the proliferation of ECs, and most importantly, the increased vascular permeability [31-33]. In our study, all three factors VEGF, VEGF1 and VEGF2 were overexpressed in psoriasis-like phenotype mice at serum, mRNA and protein levels, respectively. And this dysregulation was normalized with PF treatment. Reversed effect of PF-VEGF treatment in psoriasis mice could be a better explanation for important role of VEGF in maintaining the functioning of epidermis barrier and for a link existing between keratinocytes hyperplasia and epidermal VEGF [34]. Some studies have reported that in experimentally induced cutaneous inflammation mice VEGF was overexpressed in epidermis and induced inflammation showed resemble features as psoriasis, morphologically, histologically and immunologically [4, 35]. This provides support for the hypothesis that one of the key early features in psoriatic pathogenesis might be upregulation of VEGF and its receptors that is responsible for the epidermal and immunological changes.

The multifunctional cytokine tumor necrosis factor-alpha (TNF-α) is another regulatory factor in psoriatic angiogenesis [36]. A strong in-
crease of TNF-α was detected at both mRNA and protein levels in psoriatic mice model and significant reduction occurred with PF treatment. This trend was observed in expression levels of IL-1β, IL-6, IL-22 and IL-12 p40 in our results. It has been reported that TNF-α responsible for the upregulation of genetic transcription of VEGF [37] and increases the production of pro-inflammatory cytokines, IL-8 [38]. TNF-α was able to induce increase production of IL-6 and VEGF in HaCaT cells [39]. In a report on potential use of Cold atmospheric plasma (CAP) in the treatment of psoriasis, it is also demonstrated that CAP induced release of interleukin (IL)-6, TNF-α, VEGF and enhanced the mRNA expression of IL-1β, IL-6, IL-8, IL-10, TNF-α [40]. IL-22 is produced by T-helper 22 cells, a new subset of CD4+ cells and IL-22 is the most studied cytokine in allergic inflammatory disorders [41]. Moreover, ectopic expression of IL-12 p40 from the keratin 14 promoter led to enhanced expression of IL-23 p19 in mouse keratinocytes [42]. In this study, we elucidated the anti-inflammatory effect of PF by inhibiting the VEGF production in keratinocytes, which is mediated by a cytokine-dependent mechanism.

In this study, we propose paeoniflorin as an anti-angiogenic therapy for psoriasis. These findings might provide essential information about therapeutic effect of PF on inhibition of VEGF, particularly elucidating its anti-angiogenic function in psoriasis.

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Disclosure of conflict of interest

None.

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