Curcumin relieves TPA-induced Th1 inflammation in K14-VEGF transgenic mice

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A B S T R A C T

Curcumin has been confirmed to have anti-inflammatory properties in addition to the ability to decrease the expression of pro-inflammatory cytokines in keratinocytes. It was suggested that the interleukin-23 (IL-23)/IL-17A cytokine axis played a critical role in the pathogenesis of 12-O-tetradecanoyl phorbol 12-myristate 13-acetate (TPA)-induced K14-VEGF transgenic psoriasis-like mice model. Here, we report that topical use of a curcumin gel formulation inhibited TPA-induced Th1 inflammation in K14-VEGF transgenic mice ears but not Th17 inflammation as expected. Real-time PCR showed that mRNA levels of IL-23, IL-17A, IL-22, IL-6 and TNFα cytokines failed to increase after TPA-induction in K14-VEGF transgenic mice ear skin; but the mRNA level of IFNγ increased significantly at the same time. Furthermore, TPA-induction up-regulated the TCRγδ protein but failed to impact the CCR6 protein, which means that the proliferation of γδ T cells is incapable of IL-17A production. We find that curcumin is capable of relieving TPA-induced inflammation by directly down-regulating IFNγ production. In conclusion, curcumin inhibits TPA-induced Th1 inflammation in K14-VEGF transgenic mice which has not been previously described.

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1. Introduction

Psoriasis is a sort of immune-mediated chronic inflammatory skin disease characterized by hyperproliferative keratinocytes and the infiltration of leukocytes [1]. The most specific histopathological changes that distinguish psoriasis from other inflammatory dermatologic diseases are the dramatic hyperplasia of the epidermis with a loss of the granular layer, regular elongation of the rete ridges, thickening of the cornified layer and incomplete keratinocyte (KC) differentiation, infiltration of many different leukocytes and increased vascularity in the dermis [2]. Although the pathogenesis of psoriasis is not very clear, there is growing evidence to indicate that the dermal γδ T cells play a critical role in disease development [3–6]. Many immune-derived cytokines, including interleukin-23 (IL-23), IL-17A, IL-20, IL-22, IL-1δ, IL-6, and TNFα, are involved and interact as a network in the pathogenesis of psoriasis [3–6].

A number of animal models have been developed that possess some of the characteristic features of human psoriasis. Delivery of vascular endothelial growth factor (VEGF) to the mice’s skin results in over-expression of VEGF in epidermis, and thus produces a phenotype with more or less hallmarks of human psoriasis, such as hyper-plastic dermal blood vessels, epidermal thickening with aberrant keratinocyte differentiation, and leukocyte infiltration [7]. A mouse model with over-expression of VEGF in the epidermal basal layer of skin has been developed using a transgenic technique—the expression of murine VEGF-A164 has been combined and controlled by the human keratin 14 (K14) promoter [7]. The transgenic K14-VEGF mice can develop inflammatory skin conditions similar to human psoriasis after induction with 12-O-tetradecanoyl phorbol 12-myristate 13-acetate (TPA), characterized by increased dermal micro-vascular density and epidermal hyperplasia with aberrant keratinocyte differentiation [8]. This model is convenient for the study of anti-psoriasis therapies.

As a multi-target molecule, curcumin exhibits various pharmacological effects, such as anti-oxidant, anti-inflammatory, anti-microbial and anti-carcinogenic activities [9], that are mediated by its inhibitory effects on NF-κB, MAPK, and cytokines [10–12]. For example, curcumin down-regulates the production of many proinflammatory cytokines (such as TNFα, IL-1, IL-2, IL-6, and IL-12) by inactivating NF-κB [13,14]. Therefore, we believe that curcumin is potentially valuable for the treatment of psoriasis [15].

This study was designed to investigate the effect of a curcumin transdermal gel on a TPA-induced psoriasis-like model in transgenic K14-VEGF mice. In doing so, we expected to obtain supporting evidence for a role of curcumin in treating psoriasis.
2. Materials and methods

2.1. Chemicals

The chemicals used in this study include curcumin (Sigma, St. Louis, MO, USA), azone (Shanghai Health-well Chemical, China), TPA (Sigma, St. Louis, MO, USA), hydroxypropylcellulose (HPC)-MF (Hercules Incorporated-Aqualon Division, USA), 0.02% clobetasol propionate cream (Shanghai General Pharmaceutical Co., LTD., China), anti-RORγ antibody (Abcam, Cambridge, UK), anti-CCR6 antibody (Abcam, Cambridge, UK), anti-TCRγδ antibody (Abcam, Cambridge, UK), anti-TCRγδ antibody conjugated to biotin (Abcam, Cambridge, UK), streptavidin-peroxidase polymer (Sigma, St. Louis, MO, USA), goat anti-rabbit IgG conjugated to biotin (Santa Cruz Biotechnology, USA), and the REAL™ EnVision™ detection system using peroxidase/DAB+ and rabbit/mouse antibodies (Dako, Denmark). Mouse IL-17A/F, IL-22, IL-27 p28/IL-30 and IFNγ ELISA kits (R&D Systems, Minneapolis, USA), TRizol reagent (Invitrogen, Carlsbad, USA), and PrimeScript RT reagent kit and SYBR® premix Ex Taq™ II (Takara, Dalian, China) were also used. All other chemicals used were of analytical grade.

2.2. Preparation of curcumin transdermal gel

The 1% curcumin gel formulation used in this study was prepared from a formulation used in a previous study [15].

2.3. Transgenic animals

The K14-VEGF transgenic mice used in this study were replicated by Cyagen Biosciences Inc. according to methods described by Michael Detmar [16]. Homozygous K14-VEGF mice were used in this study.

2.4. Mice and treatment

Mice were housed under specific pathogen-free conditions and provided with food and water ad libitum. Animals were between 8 and 15 weeks old when experiments were started. Based on a previous report...

Table 1

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<thead>
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<th>Time</th>
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<td>1 day</td>
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<tr>
<td>2 days</td>
<td>Acetone + HPC gel</td>
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<tr>
<td>12 days</td>
<td>HPC gel</td>
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<tr>
<td>Control</td>
<td>TPA + HPC gel</td>
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<tr>
<td>TPA</td>
<td>TPA + HPC gel</td>
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<tr>
<td>TPA + CUR</td>
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<td>TPA + CLO</td>
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<td>CUR</td>
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TPA or acetone treatment was performed every two days and other treatments were performed twice daily. CUR, curcumin; CLO, clobetasol.

Fig. 1. Curcumin inhibited TPA-induced psoriasis-like inflammation. A, The thickness (Mean ± SD) of the right ear skin was measured on the days indicated (*P < 0.05, vs. TPA group, ANOVA followed by Student–Newman–Keuls test, N = 6). B, H&E staining of the mouse ear skin of different treatment groups (200×, N = 6). Arrowheads denote the inside of the ear skin. CUR, curcumin; CLO, clobetasol.
[8], induction with TPA started at day 0 with the application of 20 μL 0.01% TPA suspended in acetone to the inside of the right ear. TPA treatment was repeated on days 2, 4, 6 and 8. For therapy, the TPA-induction mouse model was treated twice daily with topical 50 mg/cm² curcumin HPC gel or 40 mg/cm² clobetasol propionate cream. Clobetasol was used as a positive control drug in this study. Mice smeared with acetone inside the right ear and treated similarly with HPC gel (without curcumin and azone) were used as the control group; and treated with curcumin gel as a baseline (Table 1). All protocols were approved by the experimental animal ethics committee of the Second Military Medical University and met the national guidelines for the care and use of experimental animals. The experimental animal center of the Second Military Medical University was certified by the “International Association for Assessment and Accreditation of Laboratory Animal Care International”.

2.5. Measurement of ear thickness

The ear thickness was measured using a thickness gauge (Guanglu Digital Caliper Manufacturer Co., Ltd., China) at 0, 2, 4, 6, 8, 10, 12 days. The increase in ear thickness was used to indicate the extent of inflammation. Experiments were conducted in triplicate, and the data were averaged and used to evaluate epidermal proliferation and inflammation.

2.6. Histology, immunohistochemistry and real-time quantitative PCR

At experimental day 12, ear samples from each mouse in each group were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (thickness = 4 μm) were stained using hematoxylin and eosin (H&E). Immunohistochemistry and real-time quantitative PCR were performed as described in our previous study [15]. The primer sequences used in real-time PCR are listed in Supplemental Table 1.

2.7. Measurements of RORγ, TCR γδ, CCR and cytokines in ear skin

Samples from all groups of K14-VEGF animals terminated at day 12 were investigated. To determine the levels of RORγ, TCR γδ and CCR6 expression in the mouse ear samples, the tissues were cleaned using Tris-buffered saline (TBS), cut into pieces and homogenized. Total

Fig. 2. The levels of mRNA measured using real-time PCR (Part 1). The figures show the mean value ± SD (fold) of the measured mRNA in mouse ear tissue of different treatment groups at 4 d, 8 d, and 12 days (*P < 0.05, Wilcoxon scores test, N = 6). A, IL-23. The changes in IL-23 mRNA between groups were not statistically significant. B, IL-17A. The changes in IL-17A mRNA between groups were not statistically significant. C, IL-22. The changes in IL-22 mRNA between groups were not statistically significant. D, IL-1β. The level of IL-1β mRNA in the TPA-treated group was increased significantly and was inhibited in the curcumin-/clobetasol-treated groups. E, IL-6. The changes in IL-6 mRNA between groups were not statistically significant. F, TNFα. The changes in TNFα mRNA between groups were not statistically significant. CUR, curcumin; CLO, clobetasol.
proteins were extracted and detected by Western blotting as described elsewhere [17]. Nine days following the start of experiment, the mouse auricular specimens (average weight 30 mg) were washed with PBS to remove blood, and then homogenized by a homogenizer in ice-cold PBS (0.5 mL PBS) containing a protease-inhibitor cocktail. The tissue homogenates were stored at −20 °C, and underwent two freezing and thawing cycles to disrupt the cell membrane. All tissue extracts were centrifuged (10,000 g, 4 °C) for 20 min and the supernatants were collected and assayed by ELISA. The standard protocol from the manufacturer was followed. Two experiments were pooled and data were averaged for analysis. ELISA results were normalized as pg/g tissue ultimately.

2.8. Statistics

The means and standard deviations were calculated. The significance of the differences between the treatments was determined using either ANOVA analysis or Wilcoxon scores (rank sums)/Kruskal–Wallis test. All experiments were performed in triplicate using a minimum of 3 replicates. A P-value of 0.05 was considered statistically significant.

3. Results

3.1. Effects of curcumin on TPA-induced mouse ear incrasation and inflammation

The maximum increase in ear thickness in the TPA-treated group was 0.553 ± 0.065 mm on day 10; the TPA and curcumin-treated group was 0.487 ± 0.049 mm on day 4. On day 12, the ear thickness increase in TPA and curcumin-treated group was 0.360 ± 0.045 mm, compared with the TPA-treated group (0.493 ± 0.031 mm, \(P < 0.05\)). The increase in ear thickness of the clobetasol-treated group was less than that of the TPA-treated and TPA and curcumin-treated group (between 0.315 ± 0.014 mm and 0.378 ± 0.035 mm). The ear thickness of the control group and curcumin treated control group did not show

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**Fig. 3.** The levels of mRNA measured using real-time PCR (Part 2). The figures show the mean value ± SD (fold) of the measured mRNA in mouse ear tissue of different treatment groups at 9 d (\(P < 0.05\), Wilcoxon scores test, \(N = 6\)). A, IL-27. The level of IL-27 mRNA in the TPA-treated group was increased and was inhibited in the curcumin- and clobetasol-treated groups, respectively. B, INFγ. The level of INFγ mRNA in the TPA-treated group was sharply increased and was inhibited in the curcumin- and clobetasol-treated groups, respectively. C, IL-12. The changes in IL-12 mRNA between groups were not statistically significant. D, IL-2. The changes in IL-2 mRNA between groups were not statistically significant. E, CXCL9. The level of CXCL9 mRNA in the TPA-treated group was sharply increased and was inhibited in the curcumin- and clobetasol-treated groups, respectively. F, CXCL10. The level of CXCL10 mRNA in the TPA-treated group was sharply increased and was inhibited in the curcumin- and clobetasol-treated groups, respectively. CUR, curcumin; CLO, clobetasol.
any changes during the experiment (Fig. 1A). On days 2–4 after the initiation of TPA treatment, the ear skin of the TPA-treated mice began to display signs of thickening, erythema, and mild scaling with continuously increased severity that peaked on days 6–10. Similar results were observed in the TPA and curcumin-treated groups, but the symptoms were milder than those observed in the TPA-treated group.

Results from H&E-staining of the TPA-treated ear skin showed increased subcutaneous tissue and mild epidermal thickness compared with the control group. This increased thickness was due to the increased inflammatory cells in the subcutaneous tissue. Curcumin inhibited the TPA-induced increased thickness of subcutaneous tissue, whereas clobetasol significantly inhibited the increase in thickness. No abnormal phenotype was observed in the control group and curcumin treated control group, as shown in Fig. 1B.

3.2. The mRNA levels of IL-23, IL-17A, IL-22 and other pro-inflammatory cytokines in ear samples of the TPA-induced psoriasis-like mouse model

Results from real-time PCR measurements of cytokines in ear tissue are shown in Fig. 2. In this experiment, three time points (4, 8, 12 days) were used to test for cytokine mRNA levels. Compared with the control group, the mRNA levels of IL-23, IL-17A and IL-22 changed insignificantly in all samples from TPA-treated mouse ear tissue. And the mRNA levels of IL-6 and TNFα changed insignificantly in all groups as well. However, mRNA levels of IL-1β increased mildly in TPA-treated group and could be decreased dramatically by curcumin. Similar results were observed in the clobetasol-treated group.

3.3. Curcumin decreased the high mRNA levels of IL-27, IFNγ, IL-12, IL-2, CXCL9 and CXCL10 in ear samples of the TPA-induced psoriasis-like mouse model

On day 9 after the initiation of TPA treatment, the ear tissue mRNA levels of cytokines and chemokines were measured by real-time PCR (Fig. 3). Compared with the control group, the mRNA levels of IL-27, IFNγ, CXCL9 and CXCL10 increased significantly ($P < 0.05$) in samples of TPA-treated mouse ear tissue. In contrast, the mRNA levels of IL-27, IFNγ, CXCL9 and CXCL10 significantly decreased in the curcumin-treated group when compared with the TPA-only treated group ($P < 0.05$). Similar results were observed in the clobetasol-treated group. Moreover, mRNA levels of IL-2 and IL-12 did not change significantly in all groups.

Our mRNA results showed that IFNγ was the most remarkably increased cytokine compared with IL-23, IL-17, IL-22, TNFα and IL-6 in the TPA-treated group. Meanwhile, the mRNA levels of IL-23, IL-17, IL-22, TNFα and IL-6 failed to show any meaningful changes in all groups.

3.4. ELISA detection the cytokines in skin tissue

The results from ELISA test of TPA-treated ear skin showed increasing production of IL-27 and IFNγ protein compared with other groups; both curcumin and clobetasol inhibited the TPA-induced high expression of IL-27 and IFNγ (shown in Fig. 4). In all groups, IL-17A and IL-22 expression failed to show any significant difference (shown in Fig. 4).

3.5. The impact of curcumin on RORγ, TCR γδ, CCR6 expression in skin

Results from western blotting of TPA-treated ear skin showed increasing RORγ and TCR γδ expression when compared with control group; both curcumin and clobetasol failed to inhibit the TPA-induced high expression of RORγ and TCR γδ (as shown in Fig. 5A). In all groups, CCR6 expression failed to show any significant increase and difference (shown in Fig. 5A). Immunohistochemical results from TPA-treated ear skin showed increased TCR γδ-positive cells in skin and failed to show CCR6-positive cells in all groups (shown in Fig. 5B). The immunohistochemical results agreed with results from western blotting strongly.

4. Discussion

TPA is a diester of phorbol and a potent tumor promoter often employed in biomedical research to activate the signal transduction enzyme, protein kinase C (PKC). A previous study [8] has suggested
that TPA induction leads to a Th17-like response in transgenic K14-VEGF mice. Unfortunately, it is clear that our results have reached different conclusions. Concerning differences in the methods between the studies, we support our conclusions with prudent confidence. Our results showed that the mRNA level of IL-27 was significantly increased in TPA-treated mouse ear tissue. It is well known that IL-27, a IL-12-related cytokine, has two apparently conflicting roles in the immune response: one as an initiator of Th1 responses and the other as an attenuator of inflammatory cytokine production — including IL-17A [18]. IL-27 inhibits the production of IL-17A and IL-17F in naive T cells by suppressing, in a STAT1-dependent manner, the expression of the Th17-specific transcription factor RORγ [19]. In this study, ear tissues from all TPA treated group were detected with high expression of RORγ but not CCR6. The RORγ is required for the development of several innate lymphoid populations, such as lymphoid tissue-inducer cells (LTI cells) and cells that secrete interleukin 17 (IL-17) or IL-22 [20]. It has been confirmed that CCR6 is the optimal cell surface marker for IL-17A-producing cells [3,21]. In spite of the high RORγ expression, IL-17A mRNA failed to increase accordingly which may be due to the high RORγ expression nothing on IL-17A-producing cells and influence of IL-27.

Furthermore, previous studies have shown that γδ T cells are capable of producing both IL-17A and IL-17F [22]. In murine skin, γδ T cells are abundant and act as dendritic epidermal T cells for local immune surveillance. We found mouse skin γδ TCR protein was increased following TPA treatment. However, our results show that CCR6 expression failed to be increased in all samples including TPA-treated mouse ear skin. Taken together, these results indicate that the increased γδ T cells after TPA induction in mouse skin might not be a function of IL-17A-production. Therefore, we found the conclusion that TPA induction leads to a Th17-like inflammation in K14-VEGF transgenic mice to be unsupported.

As we know, IL-27 exerts a proinflammatory Th1-enhancing activity aside from its significant anti-inflammatory functions. Murugaiyan et al. [23] have found that IL-27-induced CD4+ T cells produce large amounts of IL-10 and IFNγ. In this study, our mRNA results showed that IFNγ was the most remarkably increased cytokine after TPA-treatment compared with TNFα and IL-6, which was consistent with the previous conclusions. As CXCL9 and CXCL10 are products of IFNγ induction, their high expression strengthened the credibility of this observation as well. Although IL-2 is produced by all helper T cells early in their activation, our results showed that the mRNA levels of IL-2 and IL-12 have no significant changes after TPA induction. Additionally, evidence indicates that IL-27 limits IL-2 production during Th1 differentiation [24]. Thus, we have every reason to believe that TPA induction in K14-VEGF transgenic mice leads to a Th1 inflammation rather than a Th17-like inflammation.

Topical application of TPA on mouse skin leads to the rapid inflammatory cell infiltration and increased cytokine production. These effects make the mouse skin format symptoms closely resemble human psoriasis vulgaris with respect to erythema, skin thickening, scaling,
epidermal alteration (acanthosis, parakeratosis), and neoangiogenesis as well as the inflammatory infiltrate, including T cells and neutrophils. Although those symptoms were similar with psoriasis, their pathogenesis was different from psoriasis. Therefore, we should deliberate over the decision for using TPA-induction K14-VEGF transgenic mice psoriasis-like model to evaluate treatments for psoriasis.

Extensive scientific research over the past decade has shown that curcumin modulates multiple cellular targets and possesses therapeutic activities against a wide variety of diseases, including psoriasis [25]. In this study, we find that curcumin inhibits increased thickness and inflammation in TPA-treated mouse ear skin. The increased thickness is due to Th1 inflammation in subcutaneous tissue of the skin. Although curcumin can significantly attenuate the increase in mRNA levels of IL-27 and IFNγ after TPA-induction of this model, it remains unclear how curcumin exerts this effect. We infer that the above effects of curcumin are due to its inhibitory phosphorylation of STAT1 as a non-selective phosphorylase inhibitor (data not shown). In addition, similar effects to curcumin were observed of positive control drug — clobetasol, which just were more stronger. In conclusion, TPA-induced inflammation and this Th1 inflammation was inhibited by curcumin.

For a long time, research and evaluation of psoriasis suffered from the lack of a suitable animal model and developed slowly as a result. Therefore, we should deliberate over the decision for using TPA-induction K14-VEGF transgenic mice psoriasis-like model to evaluate treatments for psoriasis.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2015.02.007.

**References**