Curcumin induces apoptosis in tumor necrosis factor-alpha-treated HaCaT cells

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Abstract
Psoriasis is a benign, chronic skin disease characterized by keratinocyte hyperproliferation and abnormal differentiation. Curcumin, a selective phosphorylase kinase inhibitor, is a natural phytochemical present in turmeric. Curcumin has been confirmed to have anti-inflammatory properties as well as the ability to inhibit proliferation and decrease the expression of pro-inflammatory cytokines in psoriatic keratinocytes. However, the pro-apoptotic effect of curcumin in keratinocytes remains unclear. In the present study, we investigated the effect of curcumin on apoptosis induction in TNF-α-treated HaCaT cells. These results show that curcumin exhibited a significant pro-apoptotic effect on HaCaT cells only in the presence of TNF-α and/or TRAIL. The pro-apoptotic effect of curcumin resulted from the increased expression of TRAIL-R1/R2 and the decreased expression of anti-apoptotic proteins. Our results indicate that both curcumin and TNF-α up-regulated the expression of TRAIL-R1/R2. In addition, the expression of anti-apoptotic proteins (IAP1, IAP2, Bcl-XL) was up-regulated by TNF-α but suppressed by curcumin in HaCaT cells. Because these proteins are regulated by NF-κB, we examined the role of curcumin in NF-κB activation. As expected, curcumin inhibited TNF-α-induced activation of NF-κB, including NF-κB-P65. Curcumin also inhibited the TNF-α-induced production of IL-6/IL-8 in HaCaT cells. These results imply that curcumin-induced apoptosis of HaCaT cells only occurs when TNF-α or/and TRAIL are present. Therefore, we believe that curcumin is able to reverse the anti-apoptotic function of TNF-α in HaCaT cells and thus expect curcumin to be successful in the treatment of psoriasis.

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1. Introduction
Tumor necrosis factor-alpha (TNF-α) is an important cytokine that mediates immune response, inflammation and apoptosis [1]. Only a low level of TNF-α is present in the upper layer of the healthy epidermis. However, abnormal production of TNF-α is associated with a wide spectrum of diseases, including psoriasis [2,3]. Anti-TNF agents developed by rheumatologists have presented impressive effects in the treatment of severe plaque psoriasis and psoriatic arthritis and have been proven to be effective therapeutic strategies by blocking the TNF-α pathway in psoriasis [4,5]. TNF-α promotes apoptosis through binding to TNF-receptor1. However, psoriatic lesions are usually hyper-proliferative despite increased TNF-α. This paradox is partially explained by NF-κB activation, which inhibits TNF-α-induced apoptosis [6].

Curcumin is a component of turmeric and has been used as a medical remedy in Southeast Asia for centuries. This compound has anti-carcinogenic, anti-inflammatory and antioxidant properties [7-9], and evidence has shown that its anti-inflammatory role arises as a result of inhibiting NF-κB activation [10]. As a selective phosphorylase kinase inhibitor, several previous studies have shown curcumin’s potential utility in the treatment of psoriasis. Heng [11] topicaly used curcumin in the treatment of active psoriatic plaques, Pol [12] found that curcumin notably inhibited keratinocyte proliferation, and Miquel [13] reported that curcumin decreases pro-inflammatory cytokine expression in keratinocytes. However, the effect of curcumin on TNF-α-treated keratinocyte apoptosis remains unclear.

This study was designed to investigate the effect of curcumin on apoptosis in TNF-α-treated human keratinocyte cell line (HaCaT).

2. Materials and methods
2.1. Chemicals
The chemicals used in this study include TNF-related apoptosis-inducing ligand (TRAIL-R), and recombinant human TNF-α (PeproTech, London, UK); curcumin and propidium iodide (PI) (Sigma, St. Louis, MO, USA); Human Annexin V Apoptosis Detection Kit (Bender MedSystems, San Diego, USA); and human IL-6, IL-8 ELSA Testing Kit (Bender MedSystems, San Diego, USA).

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2.2. Cell culture

HaCaT cell lines were maintained and passaged in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and penicillin (100 U/mL)/ streptomycin (100 μg/mL) in our laboratory. Cultures were maintained in a humidified incubator at 37 °C with 5% CO₂.

2.3. Flow cytometric analysis of TRAIL receptors

To examine the surface staining of TRAIL receptors in HaCaT cells, flow cytometric analysis was performed as described previously [14]. Briefly, 2 × 10⁵ HaCaT cells were trypsinized, washed with PBS, and incubated with monoclonal antibodies against TRAIL-R1 to R4 or isotype-puriﬁed mouse IgG1 at 4 °C for 20 min and then with secondary biotinylated anti-mouse IgG1 antibody and phycoerythrin-labeled streptavidin under the same conditions. The working concentration of the antibodies in the experiments was 10 μg/mL. A total of 2 × 10⁴ cells were analyzed using MACSQuant® Analyzer (Miltenyi Biotec, Germany).

2.4. Apoptosis assays

Using the Annexin V Apoptosis Detection Kit combined with flow cytometry, the effect of curcumin on the induction of apoptosis in HaCaT cells was observed. After incubation with curcumin, 2 × 10⁵ HaCaT cells were harvested, washed with PBS and resuspended in 200 μL binding buffer. After the addition of 5 μL Annexin V conjugate and a 10 min incubation, the samples were resuspended in 200 μL binding buffer and 5 μL propidium iodide (PI) and analyzed using MACSQuant® Analyzer (Miltenyi Biotec, Germany). Annexin V-positive cells were designated as apoptotic cells. The data were analyzed using FlowJo software.

2.5. Western blot analysis

To determine the level of protein expression in the cytoplasm and nucleus, cell lysates were prepared as described elsewhere [15]. A total of 20–40 μg of protein was subjected to SDS-PAGE, separated by electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with primary antibody

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**Fig. 1.** Effects of curcumin on TRAIL and its receptors expression. (A) Curcumin enhanced TRAIL-R1/R2 expression. HaCaT cells were seeded in 12-well plates, incubated with 7.37 μg/mL curcumin for 4 h, and then with 20 ng/mL TNF-α for 16 h. The cell TRAIL receptors were detected by flow cytometry as described in Materials and methods section. (B) TRAIL expression. HaCaT cells were incubated with 7.37 μg/mL curcumin for 4 h, and then with 20 ng/mL TNF-α for 16 h. Whole-cell extracts were prepared and analyzed by Western blotting using an anti-TRAIL antibody. The results shown are representative of 3 independent experiments. Cur, curcumin.
followed by incubation with the species-appropriate secondary antibody coupled to horseradish peroxidase as described with minor modifications [16]. Bands were visualized using an ECL detection kit.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using nuclear extracts of HaCaT cells as described previously [17].

2.7. ELISA assay

IL-6 and IL-8 secretion levels from HaCaT cell cultures were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA). The supernatants were centrifuged to remove cell debris and stored at -80 °C before being assayed.

2.8. Statistics

All experiments were performed in triplicate with a minimum of three replicates. The means and standard deviations were calculated. The significance of the differences among the treatments was determined using either an analysis of variance (ANOVA) or the Kruskal–Wallis test. A P-value of 0.05 was considered statistically significant.

3. Results

The goal of this study was to evaluate the effect of curcumin on apoptosis induction and the inhibition of NF-κB in HaCaT cells. Because TNF-α is known to play an important role in the pathogenesis of psoriasis, cells were treated with TNF-α to examine the effect of curcumin on HaCaT cells.

3.1. Curcumin enhances the expression of TRAIL receptors in HaCaT cells

To study the effect of curcumin and TNF-α on the expression of TRAIL and TRAIL receptors, HaCaT cells were exposed to a combination treatment of 7.37 μg/mL curcumin and 20 ng/mL TNF-α for 24 h; the cells were then harvested for western blot and flow cytometric analyses. As shown in Fig. 1A, TRAIL-R1/R2 expression increased markedly after TNF-α and curcumin treatment. A similar increase in the expression of TRAIL-R3/R4 was also observed. TRAIL expression increased after treatment with both TNF-α and curcumin but remained unchanged upon single-agent treatment with either compound (Fig. 1B).

3.2. Curcumin induces apoptosis in HaCaT cells

The effect of curcumin and TNF-α on apoptosis was examined using a flow cytometric Annexin V assay. After exposure to 100, 200 and 400 ng/mL of TRAIL, the percentage of apoptotic cells fluctuated between 1.00% and 1.79%, showing insignificant differences among the experimental groups. (Fig. 2A). In another experiment, we examined the effect of curcumin on HaCaT apoptosis as modulated by TNF-α and TRAIL. A similar increase in the expression of apoptotic cells, but the combination treatment of TNF-α and TRAIL led to an increase in the percentage of apoptotic cells to 16.12%–20.83%. These results indicate that curcumin plays a pro-apoptotic role in combination with TNF-α or TRAIL in HaCaT cells.

3.3. Curcumin inhibits TNF-α-induced NF-κB activation

To investigate the effect of curcumin on TNF-α-induced NF-κB activation, HaCaT cells were pretreated with 7.37 μg/mL curcumin for 24 h and then stimulated with 20 ng/mL TNF-α for 30 min. As indicated by EMSA, TNF-α promoted NF-κB activation in HaCaT cells, while curcumin inhibited this activation. (Fig. 3A). To further examine the TNF-α-induced NF-κB activation, nuclear extracts from TNF-α-stimulated cell were incubated with a primary antibody against the p65 (RelA) subunit of NF-κB. These results suggest that the NF-κB complex inhibited by curcumin contains the p65 subunit (Fig. 3B).

3.4. Curcumin inhibits the expression of TNF-α-induced NF-κB-dependent anti-apoptotic proteins

Because apoptosis is regulated by anti-apoptotic proteins, such as IAP1, IAP2, XIAP, Bcl-XL, c-FLIP and survivin [18], we examined whether curcumin modulated the expression of anti-apoptotic proteins in HaCaT cells following TNF-α treatment (Fig. 3C). The results of western blot analysis showed that Bcl-XL, IAP1 and IAP2 expression were enhanced by TNF-α but inhibited by curcumin. In this experiment, the expression of XIAP, survivin and c-FLIP was undetected.

3.5. Curcumin inhibits TNF-α-induced IL-6 and IL-8 production in HaCaT cells

Following curcumin or TNF-α treatment, the HaCaT cell media was harvested for ELISA. These results show that curcumin markedly decreased the TNF-α-induced production of IL-6 and IL-8 in cultured HaCaT cells (Fig. 3D and E).
4. Discussion

Psoriasis is a chronic skin disease characterized by keratinocyte hyperproliferation and anti-apoptotic features [19]. Over-expression of TNF-α is a key element in pathogenesis of psoriasis vulgaris and psoriatic arthritis. Previous studies in vitro [20] show that serum or synovial fluid concentrations of IL-1, IL-6, IL-8 and TNF-α were increased in psoriasis patients. Blocking the TNF-α pathway has become an important option in the treatment of psoriasis [4,5].

A recent clinical study [21] showed that TNF-α, TRAIL-R1/R2 and TRAIL expression in psoriatic lesions was up-regulated compared with the healthy epidermis; however, the keratinocytes of psoriatic lesions show opposite anti-apoptotic features. TNF-α may contribute to this pathogenesis because, as shown herein, it enhanced the expression of anti-apoptotic proteins.

In this study, curcumin shows a pro-apoptotic effect on HaCaT cells only when combined with TNF-α treatment. Concurrently, we found that TRAIL-R1/R2 expression was significantly up-regulated by curcumin and that the increase in TRAIL expression was also modulated by the combination treatment of TNF-α and curcumin. In addition, TNF-α-induced IAP1, IAP2 and Bcl-Xl increases in expression were inhibited by curcumin. Our results clearly demonstrate that curcumin exhibits pro-apoptotic effects in combination with TNF-α. Moreover, curcumin also induces apoptosis when TRAIL is present in HaCaT cells. In another experiment, TRAIL failed to show any pro-apoptotic effects on HaCaT cells. We believe that TNF-α treatment is also responsible for this result, which leads us to the hypothesis that curcumin could reverse the anti-apoptotic features of psoriatic lesions.

TRAIL and its receptors (TRAIL-R1/R2) are well-known key regulators of cellular apoptosis. But there still had evidence to support that TRAIL sensitivity in keratinocytes is primarily regulated at the intracellular level rather than at the receptor level [22]. TRAIL-R1/R2 expression can be up-regulated by P53 [23,24] and NF-κB P65 [25], and TRAIL-R2 can also be regulated by SPI [26,27]. NF-κB P65 activity promoted by TNF-α may be responsible for the up-regulation of TRAIL-R1/R2 expression. Although TRAIL-R1/R2 and anti-apoptotic protein expression are NF-κB dependent, we observed differential responses modulated by curcumin. Therefore, we predict that curcumin and TNF-α up-regulate TRAIL-R1/R2 in different ways. More detailed studies are needed to support this hypothesis.

Additionally, our results show that curcumin inhibited TNF-α-induced NF-κB activation and the production of IL-6/8 in HaCaT cells, which is consistent with our prior conclusion [28]. Extensive evidence supporting the anti-inflammatory properties of curcumin in different cell types has previously been shown. We believe that curcumin can

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**Fig. 3.** Effects of curcumin on NF-κB, anti-apoptotic proteins and IL-6/8. (A) Curcumin inhibits NF-κB activation. HaCaT cells were incubated with 7.37 μg/mL curcumin for 4 h, and then with 20 ng/mL TNF-α for 30 min. The nuclear extracts were assayed for NF-κB activation by EMSA. (B) Curcumin inhibits nuclear translocation of P65. HaCaT cells were incubated with 7.37 μg/mL curcumin for 4 h, and then with 20 ng/mL TNF-α for 1 h. The nuclear extracts were prepared and analyzed by Western blotting using anti-P65 antibody. (C) Curcumin modulates the expression of anti-apoptotic gene products including IAP1, IAP2 and Bcl-Xl. Whole-cell extracts were prepared, and 30 μg whole-cell lysate was analyzed by Western blotting using antibodies against IAP1, IAP2, Bcl-Xl, XIAP, cFliP, and survivin as indicated. (D) Curcumin inhibits HaCaT cells to produce IL-8. HaCaT cells (2×10⁶/mL) were incubated with 7.37 μg/mL curcumin for 4 h, and then with 20 ng/mL TNF-α for 16 h. Whole-cell extracts were prepared, and 30 μg whole-cell lysate was analyzed by Western blotting using antibodies against IAP1, IAP2, Bcl-Xl, XIAP, cFliP, and survivin as indicated. (E) Curcumin inhibits HaCaT cells to produce IL-6. Cell pretreatment was the same as above. The medium was harvested for IL-6 ELISA assay. The results shown are representative of 3 or 5 independent experiments. *P<0.05 vs. the TNF group; **P<0.05 vs. the blank group. Cur, curcumin.
reduce keratinocyte-linked inflammation by inhibiting NF-κB activation. In view of report [29] on permeation study of curcumin with formulations containing of menthol as permeation enhancers, curcumin permeating through the skin and acting on its target cell are practical. Therefore, we have every reason to expect curcumin to be used in the treatment of psoriasis.

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