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Curcumin attenuates the expression of IL-1ß, IL-6, and TNF-α as well as cyclin E in TNF-α-treated HaCaT cells; NF-κB and MAPKs as potential upstream targets

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Abstract. TNF-α induces some proinflammatory cytokines including IL-1ß, IL-6, IL-8, and itself by activation of NF-κB or MAPKs (p38, JNK, ERK). These cytokines play important roles in various inflammatory skin diseases, such as psoriasis. Recently it was also reported that expression of cyclin E is up-regulated by ERK pathway after TNF-α treatment. However, it was unknown whether curcumin, showing inhibitory effects on NF-κB and MAPKs, attenuates the expression of TNF-α-induced IL-1ß, IL-6, IL-8, and TNF-α as well as cyclin E expression in HaCaT cells. In this study, we investigated the inhibitory effect of curcumin on expression of proinflammatory cytokines and cyclin E in TNF-α-treated HaCaT cells. We found that curcumin inhibited the expression of TNF-α-induced IL-1ß, IL-6, and TNF-α, but not IL-8, in TNF-α-treated HaCaT cells as well as the TNF-α-induced cyclin E expression. In addition, curcumin inhibited the activation of MAPKs (JNK, p38 MAPK, and ERK) and NF-κB in TNF-α-treated HaCaT cells. Taken together, curcumin exerts anti-inflammatory and growth inhibitory effects in TNF-α-treated HaCaT cells through inhibition of NF-κB and MAPK pathways.

Introduction

Tumor necrosis factor (TNF)-α is a key proinflammatory cytokine in various skin diseases, especially in psoriasis (1). Recently several drugs have been developed to treat psoriasis through inhibiting TNF-α (2,3). TNF-α is produced by various cells, such as macrophages, monocytes, polymorphonuclear cells, mast cells, activated T cells, and keratinocytes. Especially in TNF-α-treated keratinocytes, nuclear factor-κB (NF-κB) moves to the nucleus and mediates expression of several inflammatory cytokines, such as IL-2, IL-6, IL-8, and TNF-α itself. In addition psoriatic arthritis patients showed increased concentrations of TNF-α, IL-1, IL-6, and IL-8 in synovial fluid (4). Thus, TNF-α is a critical cytokine in psoriatic immunopathology, and development of an effective strategy to block TNF-α pathway is a key step to management of psoriasis.

Recently it was also reported that TNF-α-treated keratinocytes release epidermal growth factor receptor (EGFR) ligands including TNF-α, which induce EGFR autophosphorylation and as a consequence activate its signal transduction cascade (5). EFGR activation leads to epidermal proliferation through the persistent induction of the pathway of classical mitogen activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK) (6), and thereby resulting in promoting the accumulation of cyclins D1 and cyclin E (7). In addition p38 MAPK and c-Jun NH2-terminal kinase (JNK) are highly activated by TNF-α treatment in keratinocytes (8), and activation of p38 and JNK are associated with expression of cytokines such as IL-6 and IL-8 (9-11). Thus, blocking of TNF-α-induced MAPK pathway may result in decreasing cell proliferation as well as reducing expression of IL-6 and IL-8, thereby being a crucial point in the development of a strategy to treat psoriasis.

Curcumin is a natural phytochemical present in turmeric, the ground powder of the rhizomes of *Curcuma longa*. Curcumin has been described as having antioxidant, anti-inflammatory, and anti-carcinogenic properties (12-14). Interestingly, Kim JH *et al* reported that combination treatment with ß-phenylethyl isothiocyanate and curcumin inhibits the EGFR signaling in human prostate cancer PC-3 cells, thereby reducing cell proliferation and inducing apoptosis (15). In addition Grandjean-Laquerriere *et al* reported that curcumin attenuates the expression of ultraviolet B (UVB)-induced IL-6 and IL-8 in keratinocytes partially through inhibition of NF-κB activation (16). However it is largely unknown whether curcumin inhibits the TNF-α-induced IL-6 or IL-8 expression in HaCaT keratinocytes.

In this study we investigated whether curcumin attenuates TNF-α-induced 1L-1ß, IL-6, IL-8, or TNF-α expression through inhibition of NF-κB or MAPKs as well as the inhibitory effect of curcumin on cyclin E expression in HaCaT keratinocytes.

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Materials and methods

Materials. Antibodies against phospho-ERK (p-ERK), ERK, phospho-p38 (p-p38), p38, phospho-JNK (p-JNK), and JNK were purchased from Cell Signaling (Beverly, MA). Antibodies against tubulin and cyclin E were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and pRb antibody was purchased from PharMingen (San Jose, CA). SB203580 and SP600125 were purchased from Biomol (Plymoth, PA, CA) and Calbiochem (La Jolla, CA), respectively. Curcumin and TNF-α were purchased from Sigma-Aldrich (St. Louis, MO) and R&D Systems (Minneapolis, MN).

Cell culture. Human keratinocyte cell line HaCaT cells were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells ($5x10^4$ cells/ml) were seeded in a culture dish, and maintained in the tissue culture incubator. HaCaT cells were treated for 24 h with TNF-α ranging from 5 to 20 ng/ml, and then RNA or protein was extracted from the cells.

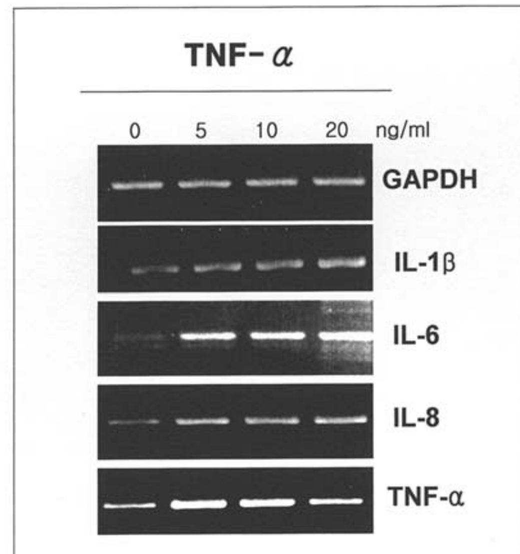

Figure 1. Up-regulation of IL-1ß, IL-6, IL-8, and TNF-α mRNA expression in TNF-α-treated HaCaT cells. The cells were cultured to 90% confluence in DMEM supplemented with 10% fetal bovine serum at 37˚C and in 5% CO_2. After TNF-α treatment for 24 h, using the indicted concentrations, cells were harvested and the cell lysates were prepared for RT-PCR analysis. Similar results were observed in two different experiments.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the RNAzol™ B (Biotecx laboratories, Houston, TX) according to the manufacturer's instructions and quantitated by spectrophotometry. One microgram of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega Co., Madison, WI). The PCR reaction was carried out under the conditions recommended by the manufacturer (Takara Co., Otsu, Japan). Briefly, 50 μl of a reaction mixture including 2.5 units of Taq polymerase (Takara Co.), 5 μl of 10X buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μl of first-strand cDNA, and 25 pmol of each primer, was subjected to 28 PCR cycles (denaturation at 94˚C for 1.5 min, annealing at 58˚C for 1 min, and polymerization at 72˚C for 1 min). The PCR products were analyzed on 1.5% agarose gel. The primer sequences and product sizes were as follows: i) GAPDH (forward, 5'-CGT CTT CAC CAC CAT GGA GA-3'; reverse, 5'-CGG CCA TCA CGC CAC AGT TT-3'), 300 base pair (bp); ii) IL-1ß (forward, 5'-AAA AGC TTG GTG ATG TCT GG-3; reverse, 5'-TTT CAA CAC GCA GGA CAG G-3'), 179 bp; iii) IL-6 (forward, 5'-GTG TGA AAG CAG CAA AGA GGC-3; reverse, 5'-CTG GAG GTA CTC TAG GTA TAC-3'), 159 bp; iv) IL-8 (forward, 5'- ATG ACT TCC AAG CTG GGC CGT G-3; reverse, 5'-TAT GAA TTC TCA GCC CTC TTC AAAA-3'), 301 bp; v) TNF-α (forward, 5'-CAA AGT AGA CCT GCC CAG AC-3; reverse, 5'-GAC CTC TCT CTA ATC AGC CC-3'), 490 bp.

Western blot analysis. Whole cell extracts were prepared in the lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, phenylmethylsulphonyl fluoride (PMSF, 10 mg/ml), aprotinin (10 mg/ml), leupeptin (10 mg/ml), 5 mM phenanthroline and 28 mM benzamidine-HCl]. For phospho-protein detection, cells were washed with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 1 mM NaF, and lysed in a buffer [20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl]. To isolate cytosolic and nuclear proteins, cells were homogenized in ice-cold hypotonic buffer (10 mM HEPES, 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, 2 mM PMSF, 1 mM DTT, 200 nM aprotinin) for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was saved as a cytosolic fraction. The pellets were homogenized in ice-cold nuclear extract buffer [10 mM Tris-Cl (pH 7.5), 0.5 M NaCl, 2.5% glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 2 mM PMSF, 200 nM aprotinin] for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was saved as a nuclear fraction. The protein concentration of extracts was estimated with Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Equal amounts of protein (40 μg/lane) were resolved by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was then washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies such as pRB (1:2000), cyclin E (1:2000), p-ERK (1:2000), ERK (1:2000), p-JNK (1:1000), JNK (1:2000), p-p38 (1:1000), p38 (1:2000), NF-κB (1:2000) and ß-tubulin (1:5000). The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Electrophoretic mobility shift assay (EMSA). For extraction of nuclear extracts, $4x10^6$ cells were washed with cold PBS,

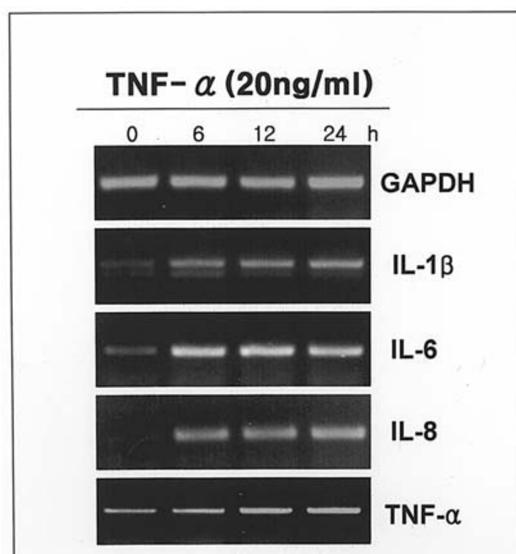

Figure 2. Up-regulation of IL-1ß, IL-6, IL-8, and TNF-α mRNA expression in TNF-α-treated HaCaT cells. The cells were cultured to 90% confluence in DMEM supplemented with 10% fetal bovine serum at 37˚C and in 5% CO_2. After TNF-α treatment (20 ng/ml), cells were harvested at the indicated times, and the cell lysates were prepared for RT-PCR analysis. Similar results were observed in two different experiments.

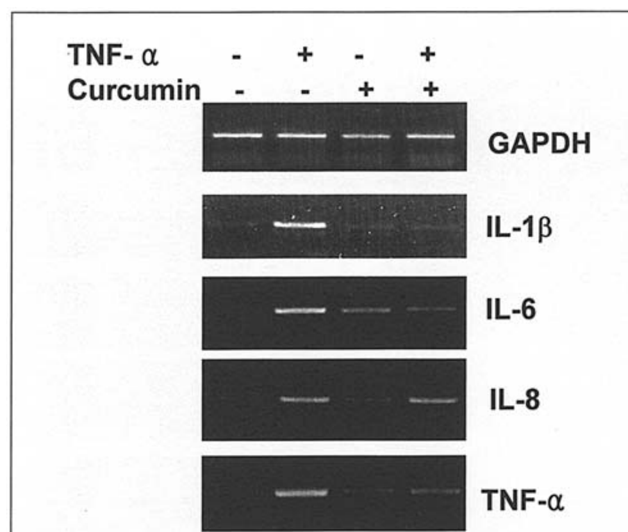

Figure 3. Inhibition of TNF-α-induced IL-1ß, IL-6, and TNF-α expression by curcumin. The cells were treated by TNF-α (20 ng/ml) with or without curcumin (20 uM) for 24 h, and cells were harvested for extraction of RNA preparation. The expression of IL-1ß, IL-6, IL-8, and TNF-α was analyzed by RT-PCR analysis. Similar results were observed in two experiments.

suspended in 400 μl of cold lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinine, 30 μg/ml Leupeptin, 5 μg/ml pepstatin, 1 mM O-phenanthroline) and incubated on ice for 15 min. Then, 25 μl of 10% NP-40 was added and the tube was vigorously mixed for 10 sec, and then the homogenate was centrifuged for 30 sec at 10,000 x g at 4˚C. The nuclear pellet was re-suspended in 50 μl of cold nuclear extraction buffer (20 mM HEPES pH 7.9, 0.4 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1000 U/ml aprotinine, 30 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM O-phenanthroline) and incubated under stirring at 4˚C for 15 min. It was then centrifuged for 5 min at 10,000 x g at 4˚C, and stored at -80˚C until use. For EMSA assay, nuclear extracts were incubated with 15 μl binding buffer (10 mM Tris pH 8, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 2.5 mM PMSF) and 50 ng of [^{32}P]-end-labelled NF-κB consensus oligonucleotide probe (5'-AGT TGA GGG GAC TTT CCC AGG C-3'). After incubation for 30 min at 20˚C, 2 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll) was added and NF-κB complexes were separated from free oligonucleotide by electrophoresis through 5% polyacrylamide gels in 0.5X TBE at 150 V for 90 min. Gels were dried and exposed to autoradiography film.

Results

Effect of TNF-α on expression of proinflammatory cytokines. To study the effect of TNF-α treatment on the expression of IL-1ß, IL-6, IL-8, and TNF-α, HaCaT cells were exposed to TNF-α at doses ranging from 5 to 20 ng/ml, and then cells were harvested 24 h after treatment for RT-PCR analysis. As shown in Fig. 1, IL-1ß, IL-6, and IL-8 expression was dramatically increased in a dose-dependent manner. Expression of TNF-α was also increased with low-dose treatment of TNF-α (5 ng/ml). Increased expression of IL-1ß, IL-6, IL-8, and TNF-α was clearly visualized at 6 h, following the sustained increased expression after TNF-α (20 ng/ml) treatment (Fig. 2). These results indicate that TNF-α induces the up-regulation of IL-1ß, IL-6, IL-8, and TNF-α in HaCaT cells.

Inhibitory effect of curcumin on TNF-α-induced expression of proinflammatory cytokines. To investigate whether curcumin inhibits the TNF-α-induced IL-1ß, IL-6, IL-8, and TNF-α expression, HaCaT cells were treated with TNF-α (20 ng/ml) with or without curcumin (10 uM). As shown in Fig. 3, up-regulation of IL-1ß, IL-6, and TNF-α by TNF-α treatment, but not IL-8, was markedly decreased by curcumin treatment, indicating that proinflammatory cytokines, such as IL-1ß, IL-6, and TNF-α expression were down-regulated by curcumin.

Effect of curcumin on TNF-α-stimulated activation of NF-κB and MAPKs. Accumulating data suggest that TNF-α-treated cells show increased expression of IL-1ß, IL-6, IL-8, and TNF-α by pathways dependent on NF-κB and MAPKs such as p38 MAPK and JNK. However it is largely unknown whether curcumin modulates the expression of IL-1ß, IL-6, and TNF-α by inhibition of NF-κB or MAPK pathways in TNF-α-treated HaCaT cells. We examined the effect of curcumin (10 uM) on activation of NF-κB, p38 MAPK and JNK in HaCaT cells. As shown in Fig. 4A, treatment of curcumin (10 uM) dramatically inhibited the activation of p65 NF-κB induced by TNF-α treatment, which was evidenced by reduced translocation of p65 NF-κB into the nucleus. Furthermore, in the presence of curcumin, TNF-induced NF-κB activation was dramatically inhibited, evidenced by decreased NF-κB binding activity (Fig. 4B). These results suggest that TNF-α-induced p65 NF-κB activation is inhibited by curcumin treatment in HaCaT cells.

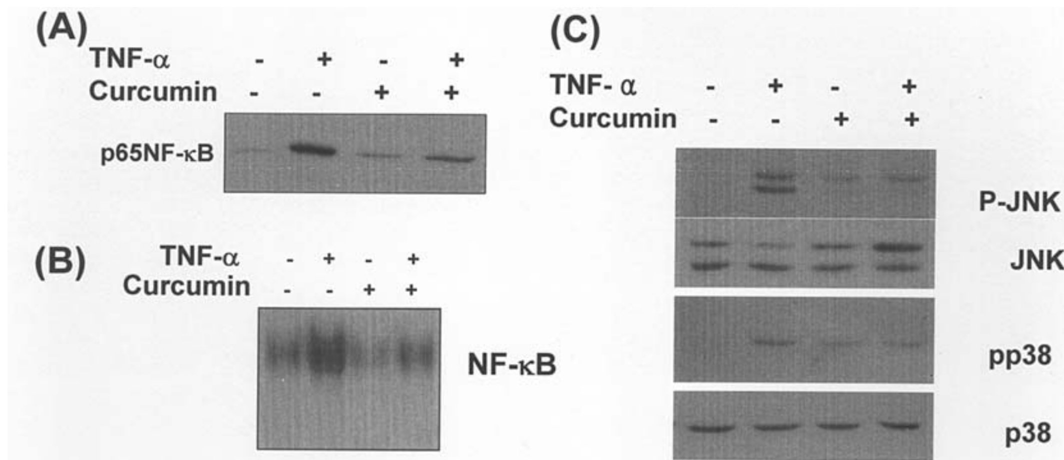

Figure 4. Inhibition of TNF-α-induced NF-κB, p38, and JNK activation by curcumin. The cells were harvested at 15 min after TNF-α treatment (20 ng/ml) with or without curcumin (20 uM), and whole cell lysates and nuclear extracts were used for Western blotting and EMSA, respectively (A and B). Whole cell lysates were also prepared 2 h after TNF-α treatment and used for p-p38 MAPK, p-JNK, p38 MAPK, or JNK Western blotting with respective antibodies (C). Similar results were observed in two experiments.

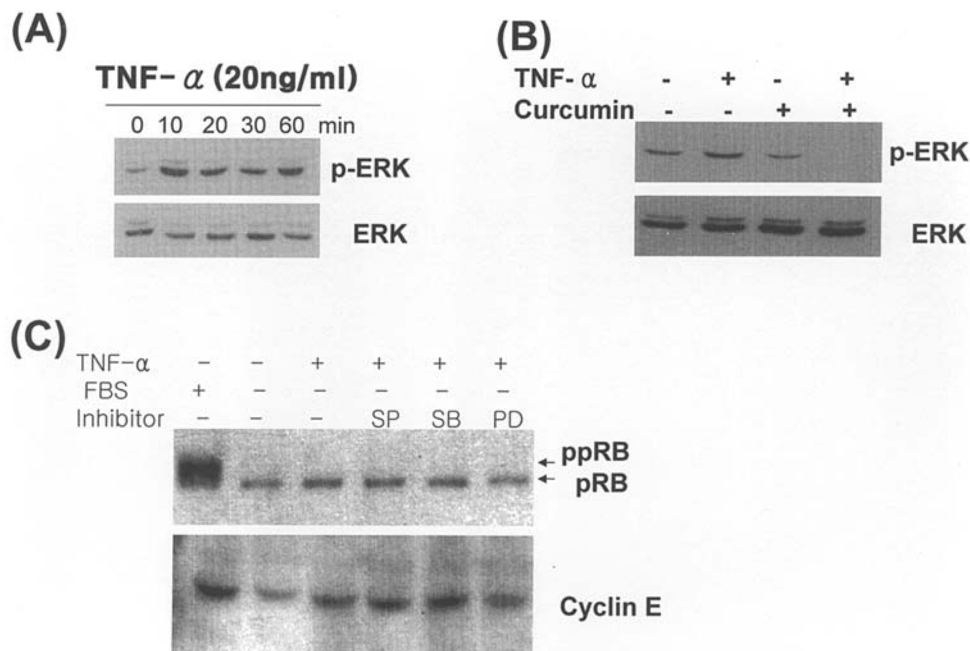

Figure 5. Attenuation of TNF-α-induced ERK activation by curcumin. The cells were harvested at 15 min after TNF-α treatment (20 ng/ml) and whole cell lysates were used for pERK and ERK (A). Whole cell lysates were also prepared 10 min after TNF-α treatment with or without curcumin (20 uM), and used for pERK and ERK Western blotting with respective antibodies (B). For cyclin E (C), TNF-α-treated HaCaT cells were cultured in the absence of FBS with ERK inhibitor (PD 98059), p38 MAPK inhibitor (SB203580), or JNK inhibitor (SP600125) in HaCaT cells for 24 h, and cell lysates were prepared and subjected to Western blotting for cyclin E and pRB. Similar results were observed in two experiments.

Activation of p38 MAPK and JNK became apparent at 5 min after treatment with TNF-α (data not shown). Stripping and reprobing the same membrane with antibodies against p38 MAPK and JNK revealed no change in total protein levels of each kinase, indicating that TNF-α induced activation of pre-existing p38 MAPK and JNK (data not shown). We next investigated whether curcumin (10 uM) inhibits the activation of p38 MAPK and JNK in TNF-α-treated HaCaT cells. As shown in Fig. 4C, phosphorylation of p38 MAPK and JNK by TNF-α was markedly decreased in curcumin-treated HaCaT cells, implying that curcumin attenuates the TNF-α-induced p38 MAPK and JNK activation in HaCaT cells. Unfortunately, we did not observe down-regulation of TNF-α-induced IL-1ß, IL-6, and TNF-α expression in HaCaT cells by pretreatment with p38 MAPK inhibitor (SB203580, 20 uM) or JNK inhibitor (SP600125, 20 uM).

Effect of curcumin on TNF-α-induced ERK activation. To investigate whether TNF-α induces the activation of ERK and inhibitory effect of curcumin on ERK, cell lysates were harvested in TNF-α-treated HaCaT cells and used for Western blot analysis. As shown in Fig. 5A, activation of ERK became

apparent at 10 min after treatment with TNF-α. Phosphorylation of ERK by TNF-α was markedly decreased in curcumin-treated HaCaT cells (Fig. 5B). Furthermore the expression of cyclin E following pRb phosphorylation, as a downstream molecule of ERK, was slightly increased by TNF-α treatment in the absence of FBS, and TNF-α-induced cyclin E expression and pRb phosphorylation were attenuated by ERK inhibitor (PD 98059) but not p38 MAPK inhibitor (SB203580) or JNK inhibitor (SP600125) in HaCaT cells (Fig. 3C), suggesting that curcumin may modulate cell proliferation by down-regulation of cyclin E expression followed by ERK in TNF-α-treated cells.

Discussion

TNF-α plays important roles in inflammatory skin diseases such as psoriasis and atopic dermatitis (17,18). Inflammatory responses to TNF-α are mediated both directly, through stimulation of the expression of IL-1, and via more distal proinflammatory cytokines, including IL-2, IL-6, IL-10, and IFN-γ (19). The expression of these inflammatory cytokines is mainly mediated by NF-κB or MAPK pathway (8,17,20). Thus, broad anti-inflammatory effects may be achieved through inhibition of TNF-α, NF-κB, or MAPK pathway.

Curcumin is known to exert its anti-inflammatory action in various cell lines through inhibition of NF-κB, AP-1, and MAPK pathways (12,16,21-23). Thus, it is important to develop an efficient strategy using curcumin to down-regulate the expression of TNF-α-induced proinflammatory cytokines. In this study, we demonstrated that curcumin attenuates the expression of TNF-α induced IL-1ß, IL-6, and TNF-α in HaCaT cells as well as inhibition of TNF-α induced NF-κB, p38 MAPK, and JNK activation. However, we did not observe p38 MAPK inhibitor (SB203580)-mediated or JNK inhibitor (SP600125)-mediated down-regulation of IL-1ß, IL-6, and IL-8 in TNF-α-treated HaCaT cells (data not shown). Thus, it seems that curcumin-induced down-regulation of IL-1ß, IL-6, and TNF-α in TNF-α-treated HaCaT cells is mediated by several combined inhibitory effects on key signal pathways including those of NF-κB and MAPKs.

Interestingly, TNF-α pathway is also associated with proliferation of human aortic smooth muscle cells (HASMC) by activation of ERK, resulting in cell cycle progression by up-regulation of cyclin D and cyclin E (24). In this study, we found that TNF-α-treated HaCaT cells showed increased expression of cyclin E in the absence of FBS, resulting in increased phosphorylation of RB protein. The proliferation rates were higher in TNF-α-treated HaCaT cells, compared to non-treated cells. Furthermore TNF-α-induced up-regulation of cyclin E was partially attenuated by ERK inhibitor PD 98059, implying that inhibition of ERK activation by curcumin may decrease the cell proliferation rates in HaCaT cells.

In conclusion, findings of the present study demonstrate for the first time, to our knowledge, that curcumin inhibits the expression of TNF-α-induced IL-1ß, IL-6, and TNF-α in TNF-α-treated HaCaT cells as well as TNF-α-induced cyclin E expression, and indicate that the inhibitory effect of curcumin on the expression of these cytokines is likely to be associated with suppression of MAPKs (JNK, p38 MAPK, and ERK) and NF-κB, thereby suggesting that curcumin may be used as a promising immunomodulatory agent in inflammatory skin diseases.

Acknowledgements

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