Inhibition of the Nuclear Factor-κB Signaling Pathway by Leflunomide or Triptolide also Inhibits the Anthralin-Induced Inflammatory Response but Does Not Affect Keratinocyte Growth Inhibition

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We performed this study to determine the relationship between activation of nuclear factor (NF)-κB and inhibition of keratinocyte growth by anthralin, which not only might be useful for a better understanding of the role of NF-κB in the pathogenesis of psoriasis, but also indicate whether the inflammatory reaction induced by anthralin is inseparable from its antipsoriatic activity. The involvement of NF-κB was assessed using the antipsoriatic drugs leflunomide and triptolide (T0) as effectors, since they can inhibit NF-κB activation induced by anthralin. The results showed that the inhibition of keratinocyte growth by anthralin was not related to the activation of NF-κB. Using sodium salicylate, a known NF-κB inhibitor, further confirmed this conclusion. Thus it might be possible to inhibit the inflammatory response induced by anthralin via repression of NF-κB activation. We found that leflunomide or T0 could significantly inhibit the mRNA overexpression of interleukin-8 and intracellular adhesion molecule-1 in keratinocytes induced by anthralin. Taken together, our data indicate that the growth inhibition of anthralin is related to the NF-κB-independent signaling pathway, and that leflunomide or T0 could control proinflammatory cytokine expression induced by anthralin via inhibiting the activation of NF-κB.

Key words nuclear factor-κB; anthralin; keratinocyte; leflunomide; triptolide

Psoriasis is a complex disorder that is characterized by abnormal epidermal proliferation and inflammation, and its pathogenesis is still not clear. Recently, many researches have indicated that the nuclear factor (NF)-κB signaling pathway is not only involved in psoriasis-related immune and inflammatory responses, but also regulates epidermopoiensis in vivo. Westergaard et al. suggested that deficient NF-κB activation in chronic psoriatic plaques contributes to the pathologic phenotype of psoriasis, implicating an inhibitory role for NF-κB activation in keratinocyte growth and epidermal thickness.

But there are other paradoxical reports. Klement et al. reported that 1κBα deficiency in epidermal cells leads to epidermal thickening and psoriasis-like lesions. Moreover, in vitro studies of human keratinocytes have shown NF-κB activation to be associated with the ability of keratinocytes to resist apoptosis induced by UV radiation. These studies support a positive role for NF-κB in regulating growth control. It is thus still unclear how NF-κB regulates epidermal thickness and keratinocyte growth.

Anthralin, which has been used for decades in the topical treatment of psoriasis, activates NF-κB in a dose-dependent fashion. Treatment of psoriatic skin with anthralin leads to the elimination of psoriatic plaques and inflammation. Previous research has suggested that anthralin induces proinflammatory molecules by activation of the transcription factor NF-κB in human keratinocytes, which leads to critical cutaneous inflammation. Some researchers hypothesized that the inflammatory reaction is necessary for the antipsoriatic activity of anthralin because derivatives of the drug that cause less irritation have a reduced antipsoriatic potential. Since it is still unclear how NF-κB regulates epidermal thickness and keratinocyte growth, that hypothesis is somewhat doubtful. To clarify the role of NF-κB activation in keratinocyte growth inhibition by anthralin might not only be useful for a better understanding of the mode of action of anthralin, but also contribute to potential therapeutic approaches to psoriasis treatment by modulating the NF-κB system.

Meanwhile, some compounds that have antipsoriatic activity such as leflunomide and triptolide (T0) have been confirmed to inhibit NF-κB activation, unlike anthralin. The inhibitory effects of leflunomide and T0 are not cell type specific. In this study, we found no correlation between the ability to activate NF-κB and inhibition of keratinocyte growth by anthralin. A second aspect was to identify whether leflunomide or T0 could affect the regulation of cytokine production mediated via activation of NF-κB by anthralin, which might be useful for designing better therapies.

MATERIALS AND METHODS

Cell Culture The spontaneously transformed human epidermal cell line HaCaT (ATCC, U.S.A.) was cultured in Dulbecco’s Modified Eagle’s medium (DMEM, Gibco, Germany) containing 10% fetal calf serum and gentamycin 40 U/l. The cells were maintained at 37 °C in a humid 5% CO2 atmosphere incubator.

Materials Leflunomide (A771726) was a gift from Cinkate Pharmaceutical Intermediates, Inc. (U.S.A.). T0 or anthralin was freshly dissolved in DMSO to a initial concentration of 10-3 M solution in DMEM and used immediately. Rabbit polyclonal antibody against 1κBα was obtained from Santa Cruz Biotechnology (U.S.A.). Goat anti-rabbit IgG conjugated to peroxidase were obtained from Boster Biotechnol-
ogy (China). TRIzol was supplied by Invitrogen Life Technologies, and AMV reverse-transcriptase and Taq DNA polymerase were provided by Promega Corporation (U.S.A.). All primers were ordered from Sangon Biotechnology (China).

Cell Extracts Cells (1.2×10^7 cells/sample) were treated with different concentrations of agents at 37 °C, and cytoplasmic extracts were prepared according to the protocol of the Nuclear Extract kit (Active Motif, U.S.A.). First, the cells were collected in ice-cold phosphate-buffered saline (PBS; KH2PO4 0.2 g, Na2HPO4 1.56 g, NaCl 8 g, KCl 0.2 g, in 11 of double distilled water, pH 7.2) in the presence of phosphatase inhibitors to limit further protein modifications. Then the cells were resuspended in hypotonic buffer to swell the membrane and render them fragile. The addition of the detergent caused leakage of the cytoplasmic proteins into the supernatant. The protein concentration of cytoplasmic proteins was measured using the method of Bradford.

Western Blotting for IκBα An equal amount of protein (100 μg/lane) from each cytoplasmic extract was submitted to electrophoresis under denaturing conditions using 10% polyacrylamide gel. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes and probed with rabbit polyclonal antibodies against IκBα at 1:200 dilution overnight at 4 °C, followed by a peroxidase-conjugated goat anti-rabbit secondary antibody. Immunoreactive bands were detected with a chemiluminescence kit (Supersignal Western Pico, Pierce, U.S.A.). Densitometric analysis was performed using a Gel Doc 2000 system with Quantity One 1-D Analysis Software (Bio-Rad, U.S.A.). Densitometric analysis was performed using a Gel Doc 2000 system with Quantity One 1-D Analysis Software (Bio-Rad, U.S.A.).

MTT Assay Cells (1×10^6) were seeded into each well of 96-well tissue culture plates. One day later, the cells were incubated in the presence or absence of the indicated test sample in a final volume of 0.2 ml at 37 °C. Thereafter, 0.02 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 4-h incubation at 37 °C, DMSO 0.15 ml was added. Optical density was measured at a wavelength of 550 nm on an Ultra Microplate reader. Cell viability was determined in six duplicate wells for each drug concentration using the MTT assay 24 h later. Growth curves were plotted based on the optical density measured 1, 2, 3, 4, 5, and 6 d later.

Reverse Transcriptase–Polymerase Chain Reaction Total cellular RNA was extracted from untreated cells or cells treated with the indicated agents according to the TRIzol protocol. The quality of the RNA was tested in 1.5% agarose gel stained with ethidium bromide (EB). The RNA zol protocol. The quality of the RNA was tested in 1.5% agarose gel stained with ethidium bromide (EB). The RNA was isolated from untreated cells or later.

Based on the optical density measured 1, 2, 3, 4, 5, and 6 d later.

RESULTS

Effects of Anthralin on Degradation of IκBα in HaCaT Keratinocytes The activation of NF-κB in response to a variety of stimuli involves its dissociation from the inhibitor IκBα and degradation of this protein via the ubiquitin-proteasome pathway. Thus the effects of anthralin on IκBα were investigated to reflect the activation of NF-κB. Western blot analysis of keratinocyte IκBα before and after treatment with different concentrations of anthralin suggested that it could induce the detectable degradation of IκBα in a dose-dependent manner (Fig. 1A). After 1 h incubation, NaSal 1 mM alone could also inhibit the degradation of IκBα significantly (Figs. 1A, B).

Effects of Leflunomide and TNα on Anthralin-Induced Degradation of IκBα It was previously shown that treatment with leflunomide 5—10 μM for 2 h suppressed TNF-induced IκBα degradation and NF-κB activation in Jurkat T

Fig. 1. Effects of Anthralin on IκBα Degradation Cells (1.2×10^7) were treated with anthralin for 2 h, and then equal amounts of cytoplasmic protein extraction were analyzed for levels of IκBα in Western blotting. The data are expressed as the percentage of IκBα levels with the control value set at 100% and are mean±S.D. of three independent experiments. *p<0.05, **p<0.01 (A). The bottom part of the panel shows results from a representative experiment (B). Unstimulated cells; 2, anthralin (50 μM); 3, anthralin (25 μM); 4, anthralin (12.5 μM); 5, anthralin (6.25 μM); 6, NaSal (1 mM).

PCR amplification of IL-8 cDNA from the RT reaction product was initiated with 2 min of denaturation at 94 °C for 1 cycle, followed by 35 cycles at 94 °C for 30 s, 55 °C for 50 s, and 72 °C for 30 s. For amplification of ICAM-1 cDNA, the RT reaction product was denatured by heating to 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 60 s, with a terminal extension at 72 °C for 7 min. After PCR amplification, the products were electrophoresed on 1.5% agarose gel and visualized by staining with EB. The size of amplified products was 289 bp for IL-8, 520 bp for ICAM-1, and 150 bp for β-actin. Band intensities were monitored by densitometric scanning and standardized against β-actin signals from parallel reactions.

Statistical Analysis Results shown are representative of three independent experiments performed in triplicate and are expressed as mean±standard deviation (S.D.). Statistical evaluation of the results was performed using the independent t-test.

RESULTS

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Effects of Leflunomide and TNα on Anthralin-Induced Degradation of IκBα It was previously shown that treatment with leflunomide 5—10 μM for 2 h suppressed TNF-induced IκBα degradation and NF-κB activation in Jurkat T
with leflunomide or T0 for 2 h can suppress anthralin-induced inflammatory response in human keratinocytes. In the present study, the results clearly showed that pretreatment with leflunomide or T0 for 2 h can suppress anthralin-induced degradation of Ikβα protein in HaCaT cells. Cells (1×10^6) were treated for 2 h with leflunomide or T0, or for 1 h with NaSal prior to a treatment with anthralin 20 μM for 2 h. Equal amounts of cytoplasmic protein extraction were prepared and analyzed in Western blotting as described in Materials and Methods for Ikβα expression. The upper part shows the mean±S.D. of three independent experiments and are expressed as the percentage of Ikβα levels with control anthralin-stimulated values set at 100%, *p<0.05, **p<0.01 (A). The lower part shows results for leflunomide (B) and T0 (C) from a representative experiment. Results are representative of three independent experiments in duplicate. B: 1, Unstimulated cells; 2, anthralin; 3, leflunomide 2.5 μM plus anthralin; 4, leflunomide 5 μM plus anthralin; 5, leflunomide 10 μM plus anthralin; 6, leflunomide 20 μM plus anthralin; 7, NaSal 1 mM plus anthralin. C: 1, Unstimulated cells; 2, anthralin; 3, T0 0.025 μM plus anthralin; 4, T0 0.05 μM plus anthralin; 5, T0 0.1 μM plus anthralin; 6, T0 0.2 μM plus anthralin.

The growth inhibition of HaCaT cells was measured daily in MTT analysis, and growth curves were established indirectly by detecting absorbence values. In the control group, HaCaT cells grew rapidly within 1—6 d. When exposed to leflunomide 20 μM or NaSal 1 mM, cell growth was slightly inhibited, and leflunomide was slightly more effective than NaSal at the indicated concentration. After being treated with T0 0.2 μM, the growth inhibition of HaCaT cells was obvious. Moreover, cell growth was completely suppressed by treatment with anthralin 20 μM (Fig. 3B).

Analysis of the Relationship between Activation of NF-κB and Anthralin-Induced Inhibition of Keratinocyte Growth  We examined the correlation between the effects of anthralin on NF-κB activation and its ability to inhibit keratinocyte proliferation. In parallel with the activation of NF-κB, pretreatment with leflunomide or T0 for 2 h was followed by the addition of anthralin 20 μM. There was no influence on anthralin-induced cell growth inhibition (Fig. 4A).

In addition, the known NF-κB inhibitor NaSal, which blocks NF-κB activation through inhibition of IKKβ phosphorylation, was employed for further confirmation of the experimental results. The addition of NaSal 1 mM 1 h before adding anthralin did not alter the anthralin-induced growth inhibition (Fig. 4B). Pretreatment with leflunomide or T0 for 4 h and 8 h, or with NaSal for 2 h, 4 h and 8 h at the indicated concentrations also did not change the results (data not shown).

Effects of Leflunomide and T0 on the mRNA Expression of Anthralin-Induced IL-8 and ICAM-1  Anthralin induces proinflammatory molecules such as IL-8 and ICAM-1 in human keratinocytes mediated by the activation of NF-κB. To determine treatment concentrations, we examined the effects of four agents on cell viability using the MTT assay. As shown in Fig. 3A, all of the compounds significantly decreased cell viability in a dose-dependent manner. Leflunomide alone up to 10^-4 M had the least effect on the cell viability of HaCaT keratinocytes. T0 was the most effective compound and decreased the viability of keratinocytes at the concentration of 10^-8 M, anthralin became effective at the concentration of 10^-6 M, while NaSal alone (up to 10^-4 M) had little effect on the viability of HaCaT cells.

The growth inhibition of HaCaT cells was measured daily
B.15) We further confirmed this in our experiment, and found that anthralin induced mRNA expression of IL-8 and ICAM-1 in a dose-dependent manner (Figs. 5A, B) when cytokine expression was investigated in parallel with the suppression of IκBα, i.e., in the activation state of NF-κB.

Leflunomide and T0 both inhibited anthralin-induced overexpression of IL-8 and ICAM-1 in a dose-dependent manner (Figs. 6A, B and Figs. 7A, B). The expression of IL-8 was reduced to about 45% and 60%, respectively, in the presence of T0 0.025 μM and leflunomide 2.5 μM, while the expression of ICAM-1 was reduced to about 70% compared with anthralin-induced cells.

DISCUSSION

Anthralin is among the most widely used drugs for the local treatment of psoriasis. Clinical treatment of psoriatic le-
sions with anthralin appears to produce relatively long remissions after cessation of therapy, although accompanied by uncomfortable skin inflammatory response.\textsuperscript{18,20} Elucidation of the therapeutic mechanisms of anthralin may help to understand the fundamental cellular alterations that cause psoriasis and be useful for designing better therapies.

Recent studies have hypothesized that NF-\(\kappa\)B activation might be related to the anthralin-induced elimination of plaques in psoriatic skin as well as the side effects of inflammatory response.\textsuperscript{15,29} We examined whether NF-\(\kappa\)B activation correlates with the inhibition of keratinocyte growth induced by anthralin. Generally, antioxidants or other NF-\(\kappa\)B inhibitors are used as effectors in such research.\textsuperscript{13,19,30} We used leflunomide and T\textsubscript{0} as effectors, which are reported to inhibit the NF-\(\kappa\)B signaling pathway in different cells and experimental conditions. Leflunomide is a pyrimidine synthesis inhibitor that has been confirmed to inhibit activation of the NF-\(\kappa\)B pathway in myeloid and epithelial cells as well as in T cell in a dose- and time-dependent manner.\textsuperscript{31} T\textsubscript{0}, derived from the Chinese herb \textit{T. wilfordii}, is known to inhibit nuclear translocation and the formation of the NF-\(\kappa\)B complex.\textsuperscript{24,35} Under our experimental conditions, leflunomide and T\textsubscript{0} were found to inhibit anthralin-mediated degradation of \(\alpha\)-Bcr in a dose-dependent manner, \textit{i.e.}, NF-\(\kappa\)B activation.

Further research showed that pretreatment with leflunomide and T\textsubscript{0} did not influence the growth of anthralin-treated cells, which means that NF-\(\kappa\)B activation was not involved in the induction of keratinocyte growth inhibition by anthralin. Pretreatment with NaSal further confirmed this. Previously, Takao \textit{et al.} clarified the relationship between NF-\(\kappa\)B activation and the inhibition of keratinocyte proliferation induced by known modulators of keratinocyte growth and concluded that keratinocyte proliferation is controlled by at least two pathways: an NF-\(\kappa\)B-dependent one and NF-\(\kappa\)B-independent one.\textsuperscript{35} Thus the mechanism of action of anthralin leading to elimination of psoriatic plaques must be related to the NF-\(\kappa\)B-independent signaling pathway, which needs to be studied further. Our results showed no evidence of modulating the NF-\(\kappa\)B system with potential for the topical treatment of psoriasis. Clearly, more work is required to clarify the precise effects of NF-\(\kappa\)B in the epidermal hyperplasia of psoriasis.

Since there is no evidence that the activation of NF-\(\kappa\)B is related to the antipsoriatic effects of anthralin, it is possible that the control of inflammatory cytokine expression induced by anthralin in keratinocytes is through selective inhibition of the NF-\(\kappa\)B signaling pathway while not reducing its antipsoriatic potential. In our studies, expression of IL-8 and ICAM-1 was significantly enhanced at the mRNA level following anthralin treatment, which is consistent with previous reports.\textsuperscript{15} Cell cultures supplemented with leflunomide or T\textsubscript{0} decreased the anthralin-mediated overexpression of the two proinflammatory cytokines. Both compounds had more effect on the overexpression of IL-8 than of ICAM-1. Previous reports showed that the regulation of cytokine gene expression is dependent not only upon activation of NF-\(\kappa\)B, but also of AP-1 nuclear transcription factor.\textsuperscript{34,35} We hypothesize that the overexpression of ICAM-1 induced by anthralin might be also regulated by AP-1 nuclear factor.

Although numerous antipsoriatic agents and therapies have been developed, all have limits in their usage.\textsuperscript{36,37} Leflunomide is highly effective in the treatment of psoriatic arthritis,\textsuperscript{38} while \textit{T. wilfordii} is in common use to treat with psoriasis punctata, psoriatic arthritis, psoriatic erythroderma, and pustular psoriasis, especially severe, chronic, or recalcitrant cases, in China. Although the two compounds have antiproliferative effects, there have been no reports on local treatment with leflunomide or \textit{T. wilfordii} or on the clinical treatment of psoriasis with \textit{T. wilfordii} except in China. We suggest that the combination of anthralin and leflunomide or T\textsubscript{0} could significantly decrease the inflammatory side effects of anthralin \textit{in vitro}, which might be useful for optimizing therapeutic outcomes.

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REFERENCES