Anti-inflammatory effects of triptolide in human bronchial epithelial cells

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BRONCHIAL EPITHELIAL CELL expression of inflammatory cytokines and growth factors contributes to the airway inflammation that is characteristic of asthma, chronic bronchitis, and bronchiectasis (reviewed in Ref. 21). Structural changes can develop in airways because of the chronic inflammation in cystic fibrosis or atypical mycobacterial pulmonary disease (11) or because of chronic allograft rejection after lung transplantation, which can manifest as obliterative bronchiolitis. Suppression of inappropriate airway inflammation by drugs delivered systemically or topically may provide therapeutic benefits in diseases such as asthma and chronic bronchitis and may help to reduce the airway destruction that occurs in bronchiectasis and obliterative bronchiolitis.

Extracts of the Chinese herb Tripterygium wilfordii exhibit potent immunosuppressive and anti-inflammatory properties and are effective traditional Chinese remedies for rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune diseases. The active compounds in the extracts of Tripterygium are all structurally related to the diterpenoid triepoxide called triptolide. Triptolide possesses antileukemic activities (12) and inhibits proliferation of transformed cell lines (20, 24). A chloroform-methanol extract of Tripterygium, T2, inhibits expression of interleukin (IL)-2 and immunoglobulin by peripheral blood lymphocytes (22). A refined extract of Tripterygium, PG27, which contains PG490 (Pharmagenesis 490, 97% pure triptolide) as its active ingredient, is an effective immunosuppressant that prolongs heart and kidney allograft survival in rat transplantation models (Fidler J, unpublished data). In addition, PG27 effectively prevents graft versus host disease in a murine model of allogeneic bone marrow transplantation (8).

Qiu et al. (18) recently described the potent inhibition of T cell activation and IL-2 gene expression by triptolide. Triptolide inhibits the transcriptional activation of the IL-2 gene by inhibiting activation of the purine-box regulator of the nuclear factor of activated T cells (NFAT) target DNA sequence in the IL-2 enhancer and by inhibiting nuclear factor-κB (NF-κB) activation. Qui et al. (18) showed that triptolide inhibits T cell activation through non-calcineurin-dependent mechanisms and that triptolide inhibits T cell activation triggered through cyclosporin A (CsA)-resistant signaling initiated at the costimulatory receptor CD28. In Jurkat T cells and A549 cells (non-small cell lung cancer), our laboratory demonstrated (13, 18) that triptolide inhibits transcriptional activation, but not DNA binding, of NF-κB. These results led us to propose that triptolide exerts its immunosuppressive effects in the nucleus by inhibiting posttranslational modifications of transcription factors such as NF-κB that regulate transcriptional activation after specific binding to DNA (18).

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NF-κB is a pleiotropic transcriptional regulator that is intimately involved in the regulation of many proinflammatory genes (reviewed in Refs. 5 and 6). NF-κB activation can promote cell survival, and NF-κB inhibition can promote apoptosis (7, 23). Through inhibition of NF-κB transcriptional activation, triptolide sensitizes tumor cells to tumor necrosis factor-α (TNF-α)-induced apoptosis of tumor cells in vitro (13). We proposed that the mechanisms of the synergy between triptolide and TNF receptor ligands in inducing apoptosis of tumor cells includes inhibition by triptolide of the survival signal conferred by NF-κB (13, 18).

In this study, we characterized the anti-inflammatory effects of triptolide in normal (NHBE) and transformed (16HBE) human bronchial epithelial cells. Triptolide potently inhibits the expression of numerous genes including cytokines, chemokines, and adhesion molecules associated with inflammation. The mechanisms of inhibition of inflammatory gene expression by triptolide include inhibition of transcriptional activation, but not DNA binding, of NF-κB. Using cDNA array hybridization, we identified bronchial epithelial cell genes involved in the inflammatory response and cell survival that are inhibited by triptolide.

**EXPERIMENTAL PROCEDURES**

*Source of triptolide.* Triptolide (PG490; mol wt 360) was obtained from Pharmacogenes (Palo Alto, CA). Triptolide appeared as white to off-white crystals at room temperature, had a melting point of 226–240°C, conformed to standard triptolide preparation as assessed by proton nuclear magnetic resonance (12), and was 97% pure by reverse-phase HPLC evaluation with acetonitrile-methanol-water (18:9:73) (Fidler J and Jin RL, personal communication).

*Cell culture and stimulation conditions.* 16HBE (16HBE140—) cells are a HBE cell line that was generated by transformation with SV40 large T antigen (9), which retains the differentiated morphology and function of normal human airway epithelia. 16HBE cells were cultured in Eagle’s MEM (Bio-Whittaker) supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO BRL, Life Technologies, Grand Island, NY). NHBE cells were purchased from Clonetics and were cultured in bronchial epithelial cell growth medium as recommended (Clonetics). Bronchial epithelial cells were stimulated in culture for the times indicated in Figs. 1–4 in the presence of phorbol 12-myristate 13-acetate (PMA; 20 ng/ml; Sigma), TNF-α (20 ng/ml; BioSource), or IL-1β (20 ng/ml; BioSource) in the absence and presence of triptolide or CsA (Sandoz).

*ELISA.* IL-8 and IL-6 protein concentrations in epithelial cell supernatants (diluted 1:10) were measured by ELISAs according to the manufacturer’s directions (Coulter).

*RNA extraction and Northern analyses.* Total RNA was isolated and analyzed by Northern hybridization as described (1, 18). cDNA probes for human IL-8 (289 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1.1 kb; Clontech) were labeled with [α-32P]dCTP (Amersham) with the use of a random hexamer labeling kit (Stratagene).

*Nuclear runoff transcription.* For a detailed description, see Ausubel et al. (4). Briefly, 16HBE cells (seven 100-mm petri dishes/condition) were either not stimulated, stimulated for 6 h with 20 ng/ml of IL-1β, or stimulated for 6 h with IL-1β in the presence of 200 ng/ml of triptolide. Cells were rinsed with PBS, scraped with a rubber policeman, and lysed two times in 4 ml of Nonidet P-40 lysis buffer A; then nuclei were resuspended in glycerol storage buffer and frozen in liquid nitrogen. Runoff transcription was performed by incubating thawed nuclei with nucleotides plus 5 μl of 10 mCi/ml of [α-32P]UTP at 30°C for 30 min. Template DNA was digested with RNase-free DNase I, RNA was extracted with phenol-chloroform-isomyl alcohol, precipitated with trichloroacetic acid-sodium pyrophosphate onto a 0.45-μm nitrocellulose filter (Schleicher and Schuell), and washed. The RNA was eluted from the filter, reextracted with phenol-chloroform, and precipitated with 3 M sodium acetate and ethanol. The precipitated RNA was resuspended, and an aliquot was used for determination of radioactivity by liquid scintillation counting. The radiolabeled RNA (1 × 106 counts/min in 1 ml for each condition) was hybridized to the IL-8 cDNA (PCR 2.1 vector containing 289-bp IL-8 cDNA (2)) that was immobilized on nitrocellulose membrane at 65°C for 36 h, then the membrane was washed with 2× saline-sodium citrate, blotted dry, and exposed to the phosphorimager screen.

*Plasmids, transfections, and luciferase reporter gene assays.* Luciferase reporter gene constructs were under the control of the IL-8 enhancer (IL-8-luciferase, nucleotides −1481 to +44 of the IL-8 enhancer (2)), the IL-8-kb site (TGGAAATTTCTCTT, IL-8-kb-luciferase), or the immunoglobulin κ light chain NF-κB sequence monomer (GGGACTTCCT) in the context of the minimal IL-8 promoter (nucleotides −45 to +44 of the human IL-8 promoter Ig NF-κB-luciferase). These plasmids also contained a neomycin resistance gene under control of the constitutively active SV40 promoter. 16HBE cells were transfected with plasmids with the use of LipofectAMINE (GIBCO BRL), and cell lines that stably expressed these luciferase reporter constructs were selected in the presence of G-418 (1.5 mg/ml). These 16HBE/IL-8-luciferase, IL-8-kb-luciferase, or Ig-NF-κB-luciferase cell lines were not stimulated or were stimulated for 6 h in the absence and presence of triptolide or CsA, and then 20 μg of whole cell extract proteins were assayed for firefly luciferase activity as described (3).

*Nuclear extract preparation and electrophoretic mobility shift assays.* 16HBE cells were stimulated for 3 h, pelleted, and washed, and then cytoplasmic and nuclear extracts were prepared as described (2). NF-κB DNA binding activities in the 16HBE cell nuclear extracts were assayed by incubating 10 μg of nuclear proteins for 30 min at 4°C in 20 μl of binding buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 10% glycerol, 50 mM KCl, and 0.05 mM dithiothreitol) containing 1–2 μg of poly(dI-dC) and 2.5 μg of [32P]-labeled oligonucleotide probe (−1 × 105 counts/min). For supershift assays, 10 μg of nuclear proteins were incubated with 2 μl of anti-NF-κB p65 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 25°C, radiolabeled probe and poly(dI-dC) were added, and the incubation was continued for an additional 30 min at 25°C. The sequence of oligonucleotide probe used was agtC- AAGAGGGACTTTCCCTAA for the immunoglobulin κ light chain NF-κB site (5). The lowercase letters indicate overhanging bases used for incorporation of [α-32P]dCTP and nonradioactive dATP, dGTP, and dTTP with Klenow DNA polymerase. Protein-DNA complexes were resolved from free probe with 4% nondenaturing polyacrylamide gels in 0.5× Tris-borate-EDTA (pH 8.3) and visualized by fluorography.

*Western immunoblotting.* The expression of IκB-α protein in 15 μg of SDS-PAGE-fractionated cytoplasmic extracts of 16HBE cells was determined by immunoblotting with a primary rabbit polyclonal IgG antibody to IκB-α (1:1,000 dilution; Santa Cruz Biotechnology), followed by a horseradish peroxidase-conjugated secondary antibody (1:3,000 dilution)
and detection with enhanced chemiluminescence (Amer- sham).

dNA array analysis. The effects of triptolide on the expression of multiple genes in 16HBE cells were analyzed with the Atlas array (Clontech) of 588 immobilized cDNAs selected for their relevance to growth control, stress responses, gene regulation, and intercellular signaling. 16HBE cells were grown to 90% confluence on 100-mm petri dishes and were either not stimulated, not stimulated and treated with triptolide (200 ng/ml) for 6 h, stimulated for 6 h with PMA (20 ng/ml), or stimulated for 6 h with PMA in the presence of triptolide (200 ng/ml). Total RNA was then isolated with RNA-STAT-60 (Tel-Test), and poly(A) mRNA was purified from 250 μg of total RNA with the use of an Oligotex mRNA mini kit (QIAGEN). The poly(A) mRNA (2 μg) from the PMA and PMA-triptolide conditions was then used as a template for reverse transcription and 32P radiolabeling (Amer sham) to generate a pool of first-strand cDNA probes according to the manufacturer’s directions (Clontech). The Atlas arrays were incubated with the radiolabeled cDNA probes, and specific binding was detected by Southern hybridization according to the manufacturer’s directions (final wash with 0.1× saline-sodium citrate-0.5% SDS at 68°C; Clontech). Specific hybridization was quantitated and analyzed with a phosphorimager with data analysis software (Cyclone, Packard Instruments). Further information about the 588 genes immobilized on the Atlas array is available at the manufacturer’s web site.

Cluster analysis. Expression-level data generated by the phosphorimager were stored in a table where the rows represented individual cDNA clones in different experimental states and the columns represented one experimental condition each. With the expression levels of the housekeeping gene α-tubulin, the raw expression levels of all the genes were adjusted between the four arrays to eliminate differences resulting from overall (background) exposure variations (Microsoft EXCEL). The adjusted expression levels were then log transformed in base 2 and median centered within each experimental condition. Average-linkage hierarchical clustering was performed with the program Cluster, and the results were analyzed with TreeView (10). The computational methods are discussed in further detail in the Cluster manual (Eisen M; http://rana.stanford.edu/software/). The color representation encodes the expression level as red with upregulation and green with downregulation. The color intensity increases with deviation from the median, with desaturated (black) areas representing no change from the median. Clusters of coregulated genes that reveal biologically interesting consequences of cell treatments with triptolide were selected for presentation as image files.

Data and statistical analyses. Significance of the differences between the experimental conditions was determined by paired two-sample Student’s t-test (Microsoft EXCEL). The data are presented as the means ± SD, and the lowest dose of triptolide that produced a significant change from the stimulated condition is identified in Figs. 1–4.

RESULTS

We demonstrated that triptolide inhibits bronchial epithelial cell IL-8 and IL-6 protein secretion triggered by diverse stimuli in NHBE and 16HBE cells. The inhibition of IL-8 mRNA expression occurs at the level of transcription. Furthermore, triptolide inhibits NF-κB transcriptional activation at a novel step after the induction of nuclear DNA binding activity.

Triptolide inhibited inflammatory cytokine gene expression by stimulated HBE cells. 16HBE cells stimulated for 12 h with PMA, TNF-α, or IL-1β markedly increased secretion of IL-8 and IL-6 protein (Fig. 1, A and D). TNF-α and IL-1β are physiological stimuli for epithelial cell inflammation, whereas PMA represents a nonphysiological, supranormal stimulus. We included PMA in our experimental protocol to discern whether the novel drug triptolide is capable of inhibiting epithelial inflammation triggered by supranormal stimuli. The induced expression of IL-8 after stimulation with PMA, TNF-α, or IL-1β involved increased expression of IL-8 mRNA (Fig. 1, B and C), suggesting that IL-8 induction involves increased gene transcription and/or mRNA stability. We show that triptolide treatment caused dose-dependent inhibition of PMA-, TNF-α-, and IL-1β-stimulated IL-8 and IL-6 protein secretion, with an IC50 of ~20 ng/ml (Fig. 1, A and D). Triptolide potently inhibited stimulated IL-8 mRNA expression while causing no significant change in GAPDH mRNA expression, suggesting that the inhibitory effect of triptolide on IL-8 and IL-6 expression is not caused by a global inhibitory effect on transcription. In contrast to these inhibitory effects demonstrated for triptolide, the immunosuppressant CsA, which blocks calcium-dependent cytokine activation in lymphocytes, produces no significant inhibition of stimulated IL-8 or IL-6 expression in 16HBE cells (Fig. 1, lanes 6 vs. 2, 12 vs. 8, and 18 vs. 14).

In NHBE cells, we show that triptolide nearly completely inhibited the IL-8 and IL-6 secretion stimulated by PMA, TNF-α-, and IL-1β, with an IC50 of 20–50 ng/ml (Fig. 1, E and F). In NHBE cells, high-dose CsA [1,000 ng/ml is 250-fold higher than the IC50 of 4 ng/ml for inhibition of peripheral blood lymphocyte secretion of IL-2 (18)] caused partial inhibition of PMA- or TNF-α-stimulated IL-8 and IL-6 secretion (Fig. 1, E and F, lanes 6 vs. 2 and 12 vs. 8). The anti-inflammatory efficacy of triptolide compared with CsA, demonstrable on epithelial cell cytokine expression, suggests that mechanisms of triptolide action are distinct from the inhibition of calcium signaling that is ascribed to CsA.

Triptolide inhibits transcriptional activation of the IL-8 gene. We performed a nuclear runoff transcription assay to demonstrate that IL-1β-stimulation of 16HBE cells results in a 1.6-fold increase in transcription of the endogenous IL-8 gene (Fig. 2A). Importantly, this increase was completely blocked when 16HBE cells were stimulated with IL-1β in the presence of 200 ng/ml of triptolide (Fig. 2A). This result indicates that triptolide inhibits IL-8 gene expression at the level of transcription.

To characterize the effects of triptolide on transcriptional induction of the IL-8 gene, we employed 16HBE cells that stably expressed a luciferase reporter gene controlled by the 1.4-kb IL-8 enhancer and promoter sequence (2). Stimulation of these 16HBE/IL-8 luciferase cells with PMA, TNF-α, or IL-1β significantly enhanced luciferase activity 1.5- to 5-fold, with PMA being the strongest stimulus, followed by IL-1β and TNF-α (Fig. 2B). The modest increases in IL-8 lucif-
erasure activation after stimulation by TNF-α or IL-1β are related to the substantial constitutive IL-8 luciferase activity present in nonstimulated 16HBE cells as described previously (2). IL-1β stimulation caused a 2.2-fold induction of IL-8 luciferase activity (Fig. 2B, lane 14), and this correlates well with the 1.6-fold induction of IL-8 transcription we observed in the nuclear runoff transcription assay (Fig. 2A). Triptolide produced dose-dependent inhibition of stimulated IL-8 luciferase activity, with an IC50 of 20–30 ng/ml (Fig. 2B). Triptolide at 200 and 1,000 ng/ml inhibited IL-8 luciferase activity to levels lower than that in nonstimulated 16HBE cells. CsA, at the high dose of 1,000 ng/ml, produced less inhibition of stimulated IL-8 luciferase activity than triptolide at 200 ng/ml (Fig. 2B).

The principal regulators of IL-8 transcriptional activation in many cell types are members of the NF-κB family, acting at the IL-8 NF-κB target DNA sequence (2, 14). We next characterized the effects of triptolide on 16HBE cells stably expressing an IL-8 NF-κB-promoter luciferase reporter construct (Fig. 2C). Similar to our results with the full IL-8 enhancer, stimulation caused a 1.4- to 5-fold induction of IL-8 NF-κB luciferase activity, which reached significance for PMA and IL-1β stimulation (Fig. 2C). Triptolide, at doses of 200 and 1,000 ng/ml, produced significant inhibition of IL-8 NF-κB luciferase activity triggered by all stimuli (Fig. 2C).

Interestingly, at the lowest dose of triptolide tested (20 ng/ml), there was superinduction of IL-8 κB luciferase activity relative to PMA and IL-1β stimuli alone (Fig. 2C, lanes 3 vs. 2 and 15 vs. 14). CsA (1,000 ng/ml) results in some inhibition of TNF-α stimulated IL-8 NF-κB luciferase activity and shows no effects on PMA or IL-1β-stimulated IL-8 NF-κB luciferase activity (Fig. 2C).

Triptolide inhibits NF-κB transcriptional activation after induction of specific DNA binding. The IL-8 NF-κB DNA target sequence may be bound by multiple nuclear factors and mediates complex regulation likely to involve both derepression and stimulation-dependent activation (14, 15, 17). To simplify our analysis of the effects of triptolide on NF-κB signaling, we chose to study DNA binding and transcriptional activation regulated by a consensus NF-κB sequence derived from the immunoglobulin κ light chain enhancer. In stimulated, triptolide-treated 16HBE cells, we correlated 1) cytoplasmic expression of the NF-κB anchoring protein IκB-α, 2) nuclear induction of NF-κB DNA binding activity, and 3) transcriptional activation of a consensus NF-κB-luciferase reporter gene (Fig. 3). Cytoplasmic IκB-α protein expression decreases markedly in 16HBE cells treated with triptolide (Fig. 3A, lanes 3–5 vs. 1). Specific DNA binding activities of NF-κB in nuclear extracts and its modulation by triptolide were characterized with the use of electrophoretic mobility

![Image](http://ajplung.physiology.org/)
shift assays. Using the consensus NF-κB oligonucleotide probe, we observed a single DNA binding complex (Fig. 2B). With the IL-8 NF-κB sequence as an oligonucleotide probe, we observed multiple specific DNA binding complexes (Aoki Y and Kao PN, unpublished observations), which serve to complicate the analysis of the effects of triptolide on NF-κB signaling. 16HBE cell stimulation with PMA, TNF-α, or IL-1β enhanced nuclear NF-κB DNA binding (Fig. 2B, lanes 2 vs. 1, 14 vs. 13, and 26 vs. 25). Triptolide treatment produced further increases in nuclear NF-κB DNA binding (Fig. 3B, lanes 3–5 vs. 2, 15–17 vs. 14, and 27–29 vs. 26). Triptolide increased nuclear NF-κB DNA binding in proportion to its inhibition of cytoplasmic IκB-α expression (Fig. 3B; compare with Fig. 3A). A specific antibody against the NF-κB p65 subunit effectively supershifted the NF-κB DNA binding complex, thereby establishing the specificity of this DNA binding complex (Fig. 3B, lanes 7–12, 19–24, and 31–36, NF-κB*).

Importantly, triptolide potently inhibited NF-κB transcriptional activation as measured with an NF-κB luciferase reporter gene in 16HBE cells (Fig. 3C). The consensus NF-κB luciferase reporter produced signals ~20-fold higher than the IL-8 NF-κB luciferase reporter in both transiently and stably transfected 16HBE cells. Cell stimulation with PMA, TNF-α, or IL-1β increased NF-κB luciferase activities between two- and fourfold (Fig. 3C), comparable to the results observed with the IL-8 NF-κB reporter (Fig. 2C). Triptolide produced dose-dependent inhibition of NF-κB luciferase activity to levels near or below those in nonstimulated cells, with an IC50 of ~50 ng/ml (Fig. 3C). Triptolide at 200 ng/ml produced greater inhibition of NF-κB luciferase activity to levels near or below those in nonstimulated cells, with an IC50 of ~50 ng/ml (Fig. 3C). Triptolide at 200 ng/ml produced near-complete inhibition of NF-κB transcription than CsA at 1,000 ng/ml (Fig. 3C). Notably, triptolide at 200 ng/ml produced near-complete inhibition of NF-κB transcription (Fig. 3C, lanes 4, 16, and 28) while simultaneously inducing strong specific NF-κB DNA binding activity (Fig. 3B, lanes 4, 16, and 28). Taken together, our results demonstrate that triptolide potently inhibited NF-κB transcriptional activation triggered by all stimuli examined at a step after specific binding to DNA.
cDNA array analysis of triptolide effects on 16HBE cell gene expression. We extended our analysis of the effects of triptolide on the expression of multiple genes in 16HBE cells using an array of immobilized cDNAs (Atlas array, Clontech). This Atlas array contains 588 genes selected for their relevance to growth control, stress responses, gene regulation, and signaling. 16HBE cells were either not stimulated, not stimulated and treated with triptolide (200 ng/ml), stimulated for 6 h with PMA (20 ng/ml), or stimulated for 6 h with PMA in the presence of triptolide (200 ng/ml). Poly(A)^+ mRNA was isolated, radiolabeled by reverse transcription, and used to probe the Atlas cDNA arrays by Southern hybridization and phosphorimerager analysis. These cell culture conditions were selected to produce supramaximal stimulation of bronchial epithelial cells and to determine the effectiveness of triptolide (applied at 4–10 times the IC50 for inhibition of 16HBE expression of IL-8) in inhibiting inflammatory gene expression.

The analysis of changes in gene expression across the four different conditions was performed by applying a clustering algorithm (10). This method is useful for extracting information that identifies genes that are similarly regulated during cellular perturbations. The phosphorimerager data of specific Southern blot hybridization intensities was median centered and converted to logarithmic scale. Then the genes with expression above the median are presented as increased intensity of red color, and genes with expression below the median are presented as an increasing intensity of green color (Fig. 4). Genes expressed at the median level appear black. We present selected strips of the cluster analysis containing regions of genes with interesting modulation by triptolide (Fig. 4). Genes with low expression in nonstimulated 16HBE cells that are induced by PMA and suppressed by triptolide are clustered as shown (Fig. 4A; green, green, red, green). Notable among these genes are cytokines, chemokines, adhesion molecules, and growth factors such as TNF-α; IL-8; MIP-2α; integrins α3, α5, α6, and β4; intercellular adhesion molecule-1 (ICAM-1); hepatoma-derived growth factor; vascular endothelial growth factor; granulocyte-macrophage colony-stimulating factor (GM-CSF); colony-stimulating factor-1; and connective tissue growth factor. Certain transcription factors cluster in a similar fashion and may regulate the ex...
expression of these genes of inflammation, including GATA-3, fra-1, Sp3, and nuclear factor NF45.

Another cluster of genes showed high expression in nonstimulated cells and PMA-stimulated cells and inhibition by triptolide. These genes cluster as shown (Fig. 4B; red, green, red, green). This cluster contains many genes associated with cell cycle regulation, including the cdc-2-related protein kinase PISSLRE, cdc protein 25, G/S-specific cyclin D1, G/M-specific cyclins A and B1, GADD-45, bel-x, and transcription factors c-jun and Jun D. These results represent the first identification of the inhibitory effects of triptolide on the expression of genes involved in cell cycle progression and survival.

The lack of global inhibitory effects of triptolide on gene expression suggests that triptolide does not operate as a nonspecific transcriptional poison. Instead, the results of the cDNA array analysis indicate that triptolide produces selective effects on gene expression that generally involve inhibition of the expression of genes associated with the inflammatory response and cell proliferation.

**DISCUSSION**

We present the first characterization of the novel anti-inflammatory mechanisms of triptolide in HBE cells. Triptolide effectively inhibits the expression of numerous genes involved in the bronchial epithelial cell inflammatory and proliferative responses.

The expression of IL-8 in bronchial epithelial cells is regulated both at the level of transcription and by posttranscriptional mRNA stabilization (19). We demonstrated that triptolide potently inhibited epithelial cell expression of IL-8 mRNA and protein. Triptolide inhibited IL-8 expression at the level of transcription, as we demonstrated using both an IL-8 nuclear run-on assay and IL-8 luciferase reporter gene experiments. Within the IL-8 enhancer, the IL-8 NF-κB target sequence contributes importantly to activation in bronchial epithelial cells (2, 19). The inhibitory effects of triptolide on the 1.4-kb IL-8 enhancer are preserved on an IL-8 NF-κB luciferase reporter (spanning nucleotides −82 to +44 of the IL-8 promoter sequence).

We performed a more detailed characterization of the effects of triptolide on DNA binding and transcriptional activation regulated by the consensus NF-κB target DNA sequence. Triptolide enhances the nuclear DNA binding activity of NF-κB in stimulated epithelial cells, likely through inhibition of IκB-α expression, which serves to release more p65 for translocation from the cytoplasm into the nucleus (18). Remarkably, triptolide enhances the nuclear DNA binding activity of NF-κB in stimulated epithelial cells, likely through inhibition of IκB-α expression, which serves to release more p65 for translocation from the cytoplasm into the nucleus (18).
Tolide inhibits NF-κB transcriptional activation even in the presence of enhanced nuclear NF-κB DNA binding activity as Qui et al. (18) showed previously in Jurkat T cells. This important result establishes that triptolide inhibits NF-κB transcriptional activation at a novel nuclear site in the signaling pathway. No other inhibitor is known to operate so distally in the NF-κB signaling pathway. Transcriptional regulation by NF-κB in the nucleus may involve coactivators, such as p300 or pCAF, that modulate chromatin conformation, in addition to interactions with the RNA polymerase II holoenzyme. The inhibitory effects of triptolide that we have observed on transiently and stably transfected reporter genes controlled by NF-κB have been observed on transiently and stably transfected holoenzyme. The inhibitory effects of triptolide that we have observed on transiently and stably transfected reporter genes controlled by NF-κB imply that triptolide inhibition does not require chromatin conformation changes. At present, we do not know the precise mechanisms of transcriptional inhibition by triptolide in the nucleus.

When we compare the IL-8 NF-κB luciferase experiments with the consensus NF-κB luciferase experiments, we note that 20 ng/ml of triptolide produces a significant enhancement of IL-8 NF-κB luciferase activity but not of consensus NF-κB luciferase activity. We never observed superinduction of the entire IL-8 enhancer luciferase reporter or enhancement of IL-8 mRNA or protein expression at any dose of triptolide. Thus superinduction of the IL-8 NF-κB luciferase reporter activity at 20 ng/ml of triptolide likely arises from the complex regulation that occurs at this non-consensus NF-κB regulatory sequence. The IL-8 NF-κB sequence bears similarity to the purine-rich/NFAT target DNA sequence in the human IL-2 enhancer (3) and has been identified as a genetic end target of the inhibitory effects of FK506 in Jurkat T cells (16). We characterized a CsA- and FK506-sensitive purine-box transcriptional regulator that is present in 16HBE cells (3) and have characterized its specific binding to an IL-8 NF-κB oligonucleotide probe (Aoki Y and Kao PN, unpublished observations). In Jurkat T cells, triptolide inhibits activation of the purine-box regulator at doses lower than those necessary for inhibition of NF-κB (18). We propose that in 16HBE cells, 20 ng/ml of triptolide primarily inhibits the purine-box regulator, thereby derepressing the IL-8 NF-κB site and superinducing transcriptional activation.

The cDNA array analysis also revealed that triptolide exerts broad inhibitory effects on the expression of genes associated with the cellular inflammatory response. Among the important genes of inflammation inhibited by triptolide are TNF-α, IL-8 and MIP-2α, and ICAM-1. The transcriptional regulation of TNF-α, IL-8, ICAM-1, and numerous other genes associated with inflammation involves regulation by NF-κB (reviewed in Ref. 5). Based on our demonstration that triptolide inhibits NF-κB signaling and transcriptional activation in the nucleus, we propose that triptolide inhibition of cytokine, chemokine, and integrin gene expression operates in part through inhibition of NF-κB transcriptional activation.

The cDNA array analysis also revealed that triptolide inhibits expression of the genes involved in cell cycle progression and cell survival. Triptolide inhibition of cyclin expression would serve to inhibit cell cycle progression and cell growth, and triptolide inhibition of bcl-x expression would promote apoptosis. In combination with ligands of the TNF receptor family, triptolide promotes apoptosis of tumor cells (13). The mechanisms underlying triptolide-induced apoptosis may involve inhibition of a survival signal conferred by NF-κB signaling as well as inhibition of survival signals associated with bcl-x expression. Furthermore, triptolide likely inhibits cell cycle progression through inhibition of the expression of cyclins D1, B1, and A1.

In summary, triptolide is a novel drug with potent anti-inflammatory and antiproliferative effects on epithelial cells. Some of the mechanisms of triptolide involve transcriptional inhibition of NF-κB signaling in the nucleus at a novel, distal site in the signaling pathway. Triptolide may be of benefit in diseases characterized by inappropriate bronchial epithelial inflammation such as the allograft rejection that follows organ transplantation and other conditions that involve airway and parenchymal lung inflammation.

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