THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 7, Issue of February 13, pp. 5877–5885, 2004

Printed in U.S.A.

Shikonins, Phytocompounds from *Lithospermum erythrorhizon*, Inhibit the Transcriptional Activation of Human Tumor Necrosis Factor α Promoter *in Vivo**

Received for publication, August 19, 2003, and in revised form, November 20, 2003
Published, JBC Papers in Press, November 25, 2003, DOI 10.1074/jbc.M309185200

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Tumor necrosis factor α (TNF-α) contributes to the pathogenesis of both acute and chronic inflammatory diseases and has been a target for the development of new anti-inflammatory drugs. Shikonins, the naphthoquinone pigments present in the root tissues of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), have been reported to exert anti-inflammatory effects both *in vitro* and *in vivo*. In this study, we evaluated the effects of shikonin and its derivatives on the transcriptional activation of human TNF-α promoter in a gene gun-transfected mouse skin system by using a luciferase reporter gene assay. The crude plant extract of *L. erythrorhizon* as well as derived individual compounds shikonin, isobutyryl shikonin, acetyl shikonin, dimethylacryl shikonin and isovaleryl shikonin showed significant dose-dependent inhibition of TNF-α promoter activation. Among the tested compounds, shikonin and isobutyryl shikonin exhibited the highest inhibition of TNF-α promoter activation and also showed significant suppression of transgenic human TNF-α mRNA expression and protein production. We demonstrated that shikonin-inhibitory response was retained in the core TNF-α promoter region containing the TATA box and a 48-bp downstream sequence relative to the transcription start site. Further our results indicated that shikonin suppressed the basal transcription and activator-regulated transcription of TNF-α by inhibiting the binding of transcription factor IID protein complex (TATA box-binding protein) to TATA box. These *in vivo* results suggest that shikonins inhibit the transcriptional activation of the human TNF-α promoter through interference with the basal transcription machinery. Thus, shikonins may have clinical potential as anti-inflammatory therapeutics.

Inflammation represents a cascade of physiological and immunological reactions as the first cellular response to noxious environmental stimuli in an effort to localize toxic materials or pathogens or to prevent tissue injury. One of the most important proinflammatory cytokines, tumor necrosis factor α (TNF-α)[1] has been shown to play a pivotal role in immune and inflammatory responses (1, 2). Inappropriate or overexpression of TNF-α is a hallmark of a number of inflammatory and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, asthma, multiple sclerosis, diabetes, and AIDS (3–9). Increased understanding of the involvement of TNF-α in the pathophysiology of various inflammatory diseases has allowed the development of new drugs that can interfere with excessive TNF-α production. Inhibition of TNF-α production or activity has been shown to be beneficial in a wide range of preclinical studies of inflammatory diseases, making inhibition of TNF-α production or signaling an appealing target for the development of novel anti-inflammatory drugs (10, 11).

Naphthoquinone compounds present in root extracts of a traditional Chinese medicinal herb, *Lithospermum erythrorhizon* Sieb. et Zucc. (LE), have been reported to confer many medicinal properties such as antibacterial, wound healing, anti-inflammatory, antithrombotic, and antitumor effects (12). Among these, shikonin, one of the active components of the roots of LE, has been shown to inhibit the capillary permeability induced by an intradermal injection of histamine and edema caused by a thermal injury to the skin of rats (13). Subcutaneous administration of shikonin has been reported to inhibit ear edema induced by croton oil in mice and paw swelling induced by yeast in rats (14). A derivative of shikonin, MDS-004, has strongly inhibited ear edema in a delayed type hypersensitivity model induced by oxazolone and dinitrofluorobenzene (15). Recent studies suggest that the anti-inflammatory effects of shikonin derivatives may be attributable to several mechanisms of action, *e.g.* inhibition of leukotriene B4 biosynthesis (14), suppression of mast cell degranulation and protection of the vasculature (16), inhibition of neutrophil respiratory burst by attenuation of protein tyrosine phosphorylation and failure of NADPH oxidase complex formation (17), impairment of phosphatidylinositol signaling (18), blockade of chemokine ligands binding to CC chemokine receptor 1 (19), and inhibition of phorbol 12-myristate 13-acetate-induced COX-2 expression (20). Although the efficacy of shikonin and its derivatives has been demonstrated *in vitro* and *in vivo*, their precise mode of action and the molecular basis for their anti-inflammatory actions *in vivo* warrants further investigation.

Skin is an immune-competent organ that serves as a first line of defense to various assaults, such as exogenous stress,

* This work was supported by Grant 91S-701-911 from the National Science and Technology Program for Agricultural Biotechnology, Taiwan, Republic of China. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TNF-α, tumor necrosis factor α; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LE, *Lithospermum erythrorhizon*; BP, *Bidens pilosa*; SH, shikonin; AS, acetyl shikonin; IBS, isobutyryl shikonin; DMAS, dimethylacryl shikonin; IVS, isovaleryl shikonin; TF, transcription factor; TBP, TATA box-binding protein; HPLC, high pressure liquid chromatography; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PBS, phosphate-buffered saline; Erk, extracellular signal-regulated kinase; EMSA, electrophoretic mobility shift assay.

environmental antigens, or pathogens. The skin immune system has been defined as a cutaneous complex of interacting immune response-related cells (21). In skin, TNF-α is a prominent cytokine that seems to be important in allergic and irritant contact dermatitis and in other inflammatory conditions (22). Modulating TNF-α expression in skin may provide therapeutic benefits for a variety of skin disorders. A simple and direct *in vivo* transfection method, particle-mediated gene transfer by gene gun, has been used for *in vivo* characterization of mammalian promoters in skin and liver tissues of rats and mice (23). In this study, we have used a gene gun-transfected mouse skin system to evaluate the effects of a crude extract and individual compounds, shikonin and its derivatives, isolated from the roots of *L. erythrorhizon* on the transcriptional activity of a transgenic human TNF-α promoter. The current study on the inhibition of TNF-α promoter activity by shikonins has provided us with an additional insight into the molecular mechanism underlying the anti-inflammatory properties of these phytocompounds. This study also demonstrated an *in vivo* quantitative molecular screening system for the evaluation of anti-inflammatory agents.

EXPERIMENTAL PROCEDURES

Mice—Female BALB/c mice (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, Republic of China) were maintained under pathogen-free conditions on standard laboratory chow and water in the animal facilities of the Institute of Biological Chemistry, Academia Sinica. All mice used in our experiments were 7–8 weeks old.

Chemicals—Hydrocortisone and betamethasone were purchased from Sigma. Chemical solutions were prepared immediately prior to the use in 70% polyethylene glycol 400 and 30% ethanol solvent. Croton oil was purchased from Fluka Chemie GmbH (Buchs, Switzerland) and diluted in acetone prior to use.

Plasmid Constructs—The pGL2 luciferase reporter vector containing the human TNF-α promoter (a generous gift from Dr. Robert L. Danner, National Institutes of Health, Bethesda, MD) was used as a template to generate a −1049 to +48 bp TNF-α promoter region flanked by MluI and BglII restriction sites using PCR and cloned into the pGL3-Basic vector (Promega, Madison, WI). The resultant plasmid was designated as pTNFP-Luc. Plasmid pIL2P-Luc was constructed by isolating a −1437 to +50 bp region of human interleukin-2 promoter flanked by MluI and BglII restriction sites from human genomic DNA through PCR and cloning into the pGL3-Basic vector. The promoterless pGL3-Basic vector was used as a negative control plasmid for luciferase assays. The plasmid pCMV-Luc containing a CMV immediate early gene enhancer/promoter has been described elsewhere (24). Clone PE4 (ATCC) was used as a template to isolate the 720-bp coding region of human TNF-α gene flanked by NcoI and XbaI restriction sites and subcloned into pTNFP-Luc by replacing the luciferase gene and designated as pTNFP-TNFC. All plasmid constructs were verified by DNA sequencing.

A series of 5′-deletions of the human TNF-α promoter were created by PCR using pTNFP-Luc (−1049/+48 bp) as template with specific 5′ primers of defined lengths relative to the transcription start site (−924/+48, −745/+48, −495/+48, −285/+48, −124/+48, and −29/+48 bp) flanked by an MluI site and a common 3′ primer flanked by a BglII site. The PCR products were cloned into pGL3-Basic vector and verified by sequencing. All plasmids used in transient transfection assays were isolated using an endotoxin-free megaplasmid purification kit (Qiagen GmbH).

In Vivo Particle-mediated Gene Transfer—The Helios gene gun system (Bio-Rad) was used to transfect the plasmid constructs into mouse skin. Plasmid DNA was precipitated onto 2-μm gold particles in the presence of spermidine and $CaCl_2$ and coated onto the inner surface of Tefzel tubing (25). The tubing was cut into 0.5-inch-length cartridges, resulting in the delivery of 0.5 mg of gold and 1.25 μg of plasmid DNA/bombardment. Female BALB/c mice were shaved on a restricted area of the abdomen and disinfected with 70% ethanol. No visual damage to epidermal skin tissue was observed. For each treatment, the target skin area was bombarded twice to deliver 2.5 μg of plasmid DNA coated onto 1 mg of gold particles with a 380 p.s.i. helium gas pressure. The bombarded area was marked with an ink stamp containing a circular sign of 2-cm diameter (3.14 cm^2).

Isolation and Administration of Herbal Compounds—The roots of the LE plants were air-dried, ground into powder, and extracted by *n*-hexane at room temperature. The solvent was removed under vacuum, and the resultant material was used as crude extract. Individual compounds were separated and purified by semipreparative HPLC (Waters HPLC system equipped with a Waters 600 controller, Waters Delta 600 pump, and 2487 Dual λ absorbance detector). A 5-μm C_{18} column (250 × 10 mm, Merck) was used with two solvent systems, acetonitrile-water (80:20, v/v) (A) and methanol (B). Elution was performed as follows: 0–8 min, A:B = 95:5 (isocratic); 8–15 min, 95–80% A to B (linear gradient); 15–40 min, 80–50% B (linear gradient) with a flow rate of 3.5 ml/min with the detection wavelength set at 254 nm. Molecular weights of isolated compounds were as follows: shikonin (SH), 288.30; acetyl shikonin (AS), 330.34; isobutyryl shikonin (IBS), 358.39; β,β-dimethylacryl shikonin (DMAS), 370.40; and isovaleryl shikonin (IVS), 372.41, as determined by mass spectrometry. Molecular structures were assigned based on various spectroscopic techniques including electron ionization mass spectrometry, Fourier transform infrared spectroscopy, and NMR analysis (Fig. 1). *Bidens pilosa* L. var. *radiata* Schult. Bip. (BP), a folk herb reputed for anti-inflammatory activities (26), was tested in parallel in this study. The dried aerial parts of BP were ground into powder and extracted by 70% ethanol at room temperature. The solvent was removed under vacuum, and the resultant material was used as a crude extract. Crude extracts and pure compounds were dissolved in an organic solvent containing 70% polyethylene glycol (polyethylene glycol 400) and 30% absolute ethanol prior to administration. A final volume of 20 μl of test extract was pipetted onto the skin area immediately after DNA transfection, spread evenly, and allowed to completely air dry. Untreated transfected skin and transfected skin treated with solvent alone, crude extract of BP, or commercial drugs were used as positive or negative controls.

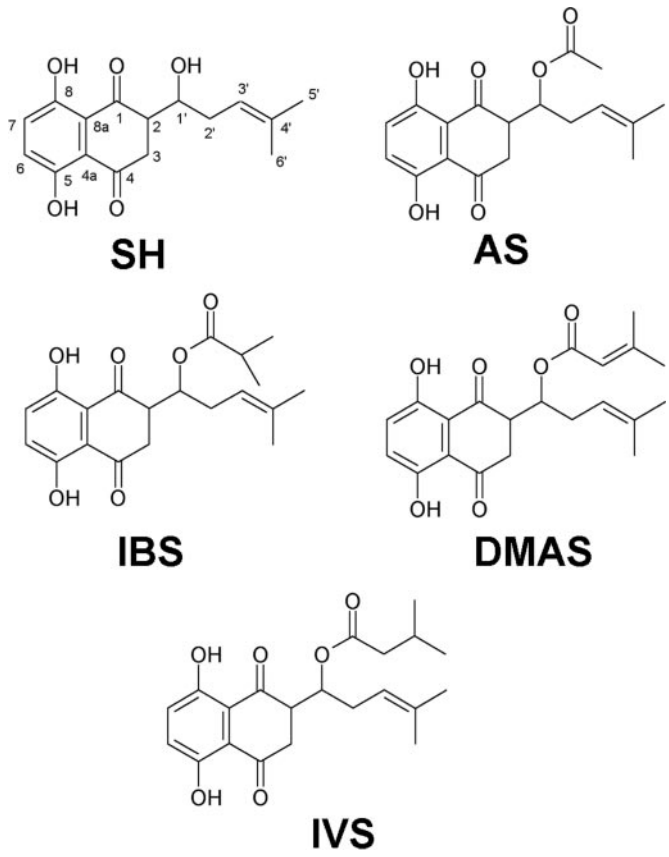

FIG. 1. **Chemical structures of the naphthoquinone compounds isolated from *L. erythrorhizon*.**

Tissue Extraction and Luciferase Assay—Animals were sacrificed 16 h post-transfection, and transfected skins (2-cm-diameter circles), treated or untreated with test agents, were removed and frozen in liquid nitrogen. Skin samples were prepared in 500 μl of lysis buffer (1× phosphate-buffered saline (PBS), 0.1% Triton X-100, and protease inhibitors) by scissor mincing followed by sonication and centrifugation of cell debris. Samples were analyzed for luciferase activity (Promega) with a Lumat LB9507 luminometer (Berthold). Duplicate analyses of two aliquots from each test skin sample were performed, and the data

were averaged. Promoter activities were measured as total relative light units/site/mouse. Induction of promoter activity was expressed as -fold increase over the control, and inhibition was expressed as percentage of the control.

RT-PCR Analysis—The expression of endogenous mouse TNF-α and transgenic human TNF-α mRNAs in mouse skin tissues was analyzed by reverse transcription-polymerase chain reaction. Frozen mouse skin samples were homogenized in liquid nitrogen. The total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and resuspended in 25 μl of diethyl pyrocarbonate-treated water. RT-PCRs were carried out by using the AccessQuick RT-PCR system (Promega) according to the manufacturer's instructions. Briefly 1 μg of total RNA from each sample was added to the reaction mixture containing 1× AccessQuick master mixture (*Tfl* DNA polymerase, avian myeloblastosis virus/*Tfl* reaction buffer, 25 mM $MgSO_4$, and 10 mM dNTP mixture), a 10 μM concentration of each of specific sense and antisense primers, 5 units of avian myeloblastosis virus reverse transcriptase, and nuclease-free water to obtain a final volume of 50 μl. Reactions were incubated at 48 °C for 60 min, and PCR amplification was carried out after denaturing at 95 °C for 2 min. The primers contained the following sequences: mouse TNF-α sense primer, 5′-ATGAGCACAGAAAGCATGATCCGCGACG-3′, and antisense primer, 5′-GACTCCAAAGTAGACCTGCCCGGACTC-3′; mouse GAPDH sense primer, 5′-CATCACTGCCACCCAGAAGACTGTGGA-3′, and antisense primer, 5′-TACTCCTTGGAGGCCATGTAGGCCATG-3′; human TNF-α sense primer, 5′-GAAAGCATGATCCGGGACGTGGA-3′, and antisense primer, 5′-GTTGGATGTTCGTCCTCCTCACA-3′. The PCR products were separated on 1.5% agarose gels (Ultrapure, Invitrogen) at 55 V for 75 min along with a molecular weight marker, GeneRuler 100-bp DNA Ladder (MBI Fermentas) and visualized by UV illumination after staining with 0.5 μg/ml ethidium bromide solution. Gels were photographed with type 55 positive/negative film (Polaroid Corp., Cambridge, MA). Images were scanned, and densitometry analysis of the captured image was performed using BIO-1D image analysis software. The signal intensities of the test genes in different samples were normalized to the respective mouse GAPDH signal intensity.

ELISA for TNF-α—Endogenous mouse TNF-α and transgenic human TNF-α cytokine concentrations in mouse skin tissues were determined using OptEIA mouse TNF-α and OptEIA human TNF-α ELISA kits, respectively (Pharmingen). The capture and detection antibodies used were specific for mouse and human TNF-α cytokines. Briefly skin samples were prepared in 1 ml of lysis buffer as described for the luciferase assay. 96-well plates were coated with 100 μl/well anti-mouse or anti-human TNF-α capture antibody (1:500) and incubated overnight at 4 °C. Wells were washed three times with wash buffer (PBS with 0.05% Tween 20). Plates were blocked with assay diluent (PBS saline with 10% fetal bovine serum, pH 7.0) for 1 h and then washed. 100 μl of each respective standard, samples, and controls were added, and plates were incubated overnight at 4 °C. After five washes, 100 μl of working detector (biotinylated anti-mouse TNF-α polyclonal (1:500) or biotinylated anti-human TNF-α monoclonal antibody (1:500) and avidin-horseradish peroxidase) was added, and plates were incubated for 1 h at room temperature. After a final wash, tetramethylbenzidine and hydrogen peroxide substrate solution was added and incubated for 30 min at room temperature in the dark. Absorbance was read at 450 nm in an ELISA reader after the addition of stop solution. No cross-reactivity was observed between mouse and human TNF-α cytokines.

Western Blot Analysis—Mice were bombarded with gold particles alone or pTNFP-Luc DNA-coated gold particles on their shaven abdomens as described above. Transfected skin was untreated, treated with solvent alone, or treated with shikonin, and skin samples were collected at the indicated time points. For isolation of total protein, mouse skin was excised, immediately placed in liquid nitrogen, and pulverized in mortar. The pulverized skin was lysed in 2 ml of ice-cold lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 20 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, and protease inhibitor mixture tablets) for 10 min. Lysates were centrifuged at 12,000 × *g* for 20 min, and supernatant containing 30 μg of protein was boiled in SDS sample loading buffer for 10 min before electrophoresis on a 12% NuPAGE BisTris gel (Invitrogen). After electrophoresis for 2 h, proteins in the gel were transferred to polyvinylidene difluoride membrane (Novex, San Diego, CA), and the blots were blocked with 5% nonfat dry milk, PBST buffer (PBS containing 0.1% Tween 20) for 60 min at room temperature. The membranes were incubated overnight at 4 °C with a 1:1000 dilution of phospho-p44/42 mitogen-activated protein kinase (extracellular signal regulated-kinase 1/2 (Erk1/2)) and phospho-NF-κB p65 polyclonal antibodies (Cell Signaling Technology Inc., Beverly, MA). Equal protein loading was assessed using mouse β-actin (Sigma). The blots were rinsed three times with PBST buffer for 5 min each. Washed blots were incubated with a 1:2000 dilution of the horseradish peroxidase-conjugated secondary antibody and then washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

Electrophoretic Mobility Shift Assay—Mice were treated as described for Western blotting, and skin samples were collected after 1 h. For isolation of nuclear protein, mouse skin was excised, immediately placed in liquid nitrogen, and pulverized in mortar. The pulverized skin was lysed in 2 ml of ice-cold hypotonic buffer (10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride for 15 min on ice. To the lysates, 125 μl of 10% Nonidet P-40 solution was added, and the mixture was centrifuged for 2 min at 14,800 × *g*. The pelleted nuclei were washed once with 400 μl of buffer A plus 25 μl of 10% Nonidet P-40, centrifuged, and resuspended in 150 μl of nuclear extract buffer (50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) for 30 min. The mixture was centrifuged for 10 min at 4 °C. The supernatant containing nuclear proteins was collected, and protein concentrations were determined. EMSA was performed using a LightShift chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocol. Briefly double-stranded oligonucleotide corresponding to the −30 bp core promoter element of human TNF-α, 5′-GGACATATAAAGGCAGTTGTTGGCACACCC-3′ (−30 to −1) was end-labeled with biotin (Purigo Biotech, Taipei, Taiwan). Binding reactions were carried out in a total volume of 20 μl containing 1× binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol, pH 7.5), 50 ng/μl poly(dI·dC), 2.5% glycerol, 0.05% Nonidet P-40, 5 mM $MgCl_2$, 5 μg of nuclear proteins, and 20 fmol of labeled probe. A 200-fold excess of unlabeled oligonucleotide (competitor) was added where necessary. Where direct binding of shikonin with the probe was tested, nuclear protein was replaced by shikonin in binding reactions. After a 20-min incubation at room temperature, 5 μl of loading buffer was added, and samples were electrophoresed through a 6% native polyacrylamide gel at 100 V. Electrophoresed binding reactions were transferred to nylon membrane and detected by following the manufacturer's instructions.

Statistical Analysis—Results are expressed as mean ± S.D. Statistical differences were assessed with an unpaired, two-tailed Student's *t* test.

RESULTS

Transcriptional Activity of Transgenic Human TNF-α Promoter in Mouse Skin Tissue—To develop an *in vivo* molecular screening system for the evaluation and identification of anti-inflammatory phytocompounds, we transfected a proinflammatory cytokine human TNF-α promoter (−1049/+48 bp)-luciferase reporter construct (pTNFP-Luc) into mouse skin via particle-mediated gene transfer using a Helios gene gun. Transgenic promoter activity was measured in terms of luciferase activity expressed in relative light units/defined tissue site (3.14 cm^2/mouse). We observed a relatively high level of human TNF-α promoter activity, which was 2370-fold higher than the promoterless negative control vector (pGL3-Basic) and was only 8.4-fold lower than that obtained for a constitutively active CMV immediate early gene enhancer/promoter (pCMV-Luc) in mouse skin (Fig. 2), whereas the luciferase activity obtained from skin samples transfected with pIL2P-Luc vector containing the human interleukin-2 cytokine promoter (−1437/+50 bp) was less than 1% of that observed for the human TNF-α promoter (pTNFP-Luc).

Induction of Local Inflammatory Response and Endogenous TNF-α Expression in Mouse Skin Tissue by Gene Gun Particle-mediated Physical Injury/Stress—The relatively high level of human TNF-α promoter activity observed in mouse skin tissue led us to suspect that gene gun particle-mediated physical injury/stress may have resulted in its transcriptional activation. To test this hypothesis, mouse skin tissue was bombarded twice to deliver 1 mg of 2-μm gold particles, without any coated DNA, by using the gene gun at 380 p.s.i. helium gas pressure/bombardment. As shown in Fig. 3*A*, within 1 min of particle bombardment we observed reddening of skin resulting from an

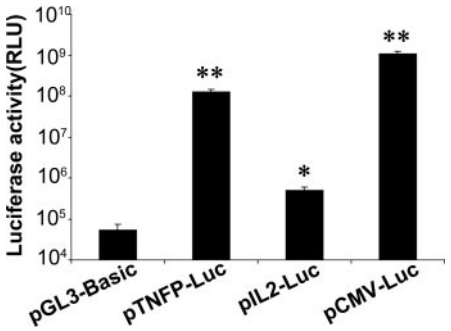

FIG. 2. **Transcriptional activity of human TNF-α promoter in mouse skin tissues.** The test reporter plasmid pTNFP-Luc contained a portion of the human TNF-α promoter (−1049 to +48 bp) ligated to the luciferase reporter gene. Promoterless luciferase reporter vector pGL3-Basic served as negative control, and plasmid pIL2P-Luc containing human interleukin-2 promoter (−1437 to +50 bp) and cytomegalovirus immediate early enhancer/promoter-luciferase construct (pCMV-Luc) were used as positive controls. Plasmids were transfected into mouse abdominal skin at a dose of 2.5 μg/site/mouse by gene gun bombardment. Skin samples were harvested and processed 16 h post-transfection and assayed for luciferase activity as described under "Experimental Procedures." The mean luciferase activities obtained from three independent transfections of a representative experiment are shown (±S.D.). *, $p < 0.05$ and **, $p < 0.01$ *versus* promoterless negative control vector. *RLU*, relative light units.

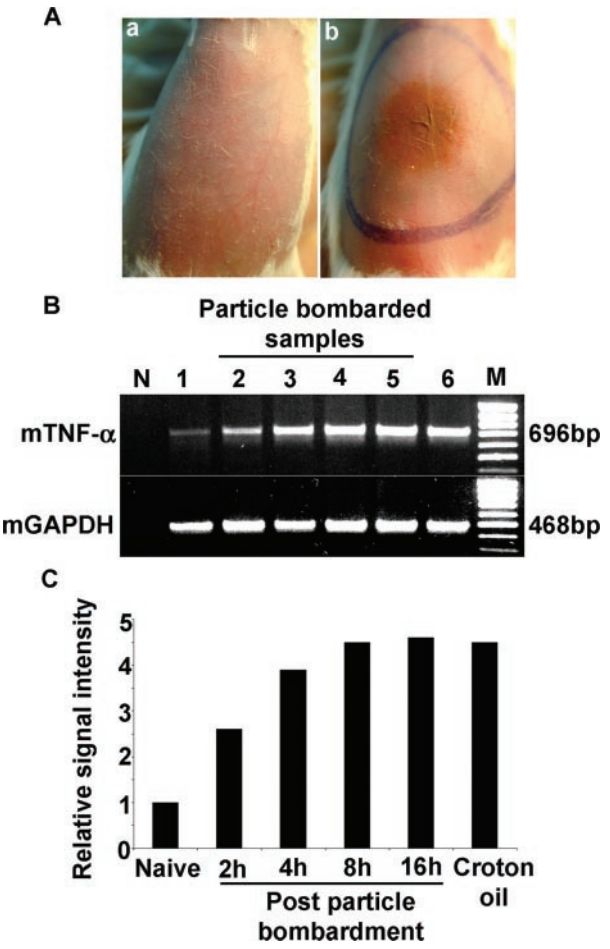

FIG. 3. **Induction of local inflammatory response and endogenous TNF-α mRNA expression in mouse skin tissues by gene gun-generated physical injury/stress.** Mouse abdominal skin was either not bombarded or bombarded with gold particles, and total RNA was extracted from tissues and analyzed by RT-PCR as described under "Experimental Procedures." *A*, erythema observed on mouse abdominal skin. *a*, unbombarded skin; *b*, gold particle-bombarded skin. *B*, electrophoresis pattern of PCR products. *Lane N*, no template; *lane 1*, unbombarded skin; *lanes 2*, *3*, *4*, and *5*, skin samples collected at 2, 6, 8, and 16 h after gold particle bombardment, respectively; *lane 6*, 2% croton oil-treated sample; *lane M*, DNA size marker. *C*, densitometry analysis. The signal intensity of the test gene and GAPDH were quantified using an image analyzer. The changes in the signal intensity of the test gene relative to GAPDH were calculated and expressed as -fold of unbombarded control. *m*, mouse.

acute local inflammatory response (erythema). Skin samples were collected at different time points, and endogenous mouse TNF-α mRNA expression levels were analyzed by reverse transcription-PCR analysis. A time-dependent induction of endogenous TNF-α expression was observed in gold particle-bombarded skin samples (*lanes 2–5*) as compared with unbombarded control skin (*lane 1*) (Fig. 3*B*). As a positive control (*lane 6*), topical application of 2% croton oil (non-sensitizing contact irritant) also induced clearly detectable endogenous mouse TNF-α mRNA expression. Densitometry analysis showed a 4.6-fold increase in TNF-α mRNA expression level at the 16-h time point (*lane 5*) after gold particle bombardment as compared with unbombarded control skin (*lane 1*) (Fig. 3*C*). We further analyzed the endogenous mouse TNF-α protein production in response to gene gun particle-mediated physical injury/stress by using a mouse-specific ELISA kit. Gold particle-bombarded skin samples showed a 6.0-fold increase in TNF-α protein production as compared with unbombarded control skin (Table I). These results suggested that gene gun particle-mediated physical injury/stress could induce localized inflammatory response, activate TNF-α promoter, and induce its mRNA expression and protein production. Therefore, this indicated that the particle-mediated physical injury/stress-induced human TNF-α promoter activity in mouse skin tissue could be used as a base-line control to compare the inhibitory effects of potential anti-inflammatory agents.

Crude Extract of LE and Topical Anti-inflammatory Drugs Inhibit the Transcriptional Activity of Human TNF-α Promoter in Vivo—To study the effect of LE crude extract on the transcriptional activation of TNF-α promoter and compare its effect with that of the commercially available topical anti-inflammatory drugs hydrocortisone and betamethasone, we transfected mouse abdominal skin with plasmid pTNFP-Luc via gene gun delivery. Transfected skin was treated with 100 μg of each test agent/site/mouse and a crude extract of BP as a negative control treatment. 16 h after treatment, skin samples were assayed for reporter gene activity. Crude extract of LE and topical anti-inflammatory drugs hydrocortisone and betamethasone significantly inhibited the transcriptional activity of TNF-α promoter as compared with the untreated control in our *in vivo* system (Fig. 4), whereas treatment with crude extract of BP did not show any effect on TNF-α promoter activity. The inhibitory effect of crude LE (49%) on TNF-α promoter activity was comparable to that of hydrocortisone (60%) and betamethasone (44%).

TABLE I
Induction of endogenous mouse TNF-α protein production by gene gun-generated physical injury/stress

Treatment	pg/ml	-Fold induction
No bombardment	10.2 ± 0.75	1
Gold particle bombardment	62.1 ± 3.72[a]	6.0

[a] $p < 0.01$.

Shikonin Compounds Inhibit the Transcriptional Activity of Human TNF-α Promoter in Vivo—Five pure compounds, SH, DMAS, AS, IBS, and IVS, were isolated from *L. erythrorhizon.* To investigate which compound(s) was contributing to the observed inhibitory effect of crude LE extract on transcriptional activity of TNF-α promoter, plasmid pTNFP-Luc-transfected skin was treated with each compound individually and also with the crude extract at concentrations of 50, 100, and 200

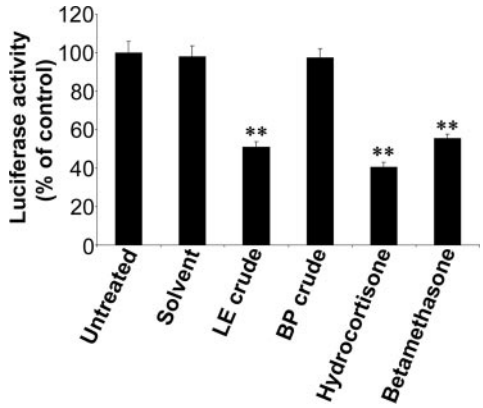

FIG. 4. **Inhibition of transcriptional activation of human TNF-α promoter by crude extract of LE, hydrocortisone, and betamethasone.** Mouse skin was transfected with pTNFP-Luc construct, and transfected skins were either untreated, treated with solvent, or treated with 100 μg of each of the test agents/site/mouse, namely LE crude extract, BP crude extract, hydrocortisone, and betamethasone. Skin samples were harvested and processed as described in Fig. 2. Luciferase activity of treated samples was expressed as percentage of the untreated control. The mean values obtained from three independent transfections of a representative experiment are shown (±S.D.). **, $p < 0.01$ *versus* untreated control.

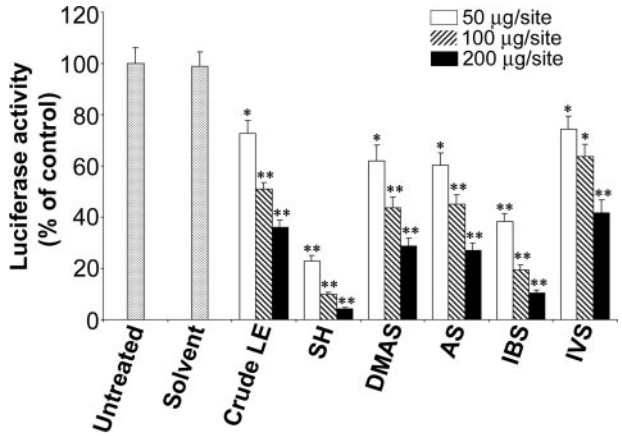

FIG. 5. **Inhibition of transcriptional activation of human TNF-α promoter by individual pure compounds of LE.** Mouse skin transfected with pTNFP-Luc construct was treated with crude extract of LE and its derivatives SH, DMAS, AS, IBS, and IVS, each at three different concentrations (50, 100, and 200 μg/site/mouse). Transfected skins, either untreated or treated with solvent (70% polyethylene glycol + 30% ethanol), were used as controls. Skin samples were harvested and processed as described in Fig. 2. Luciferase activity of treated samples was expressed as percentage of the untreated control. The mean values obtained from three independent transfections of a representative experiment are shown (±S.D.). *, $p < 0.05$ and **, $p < 0.01$ *versus* untreated control.

μg/site/mouse. Samples were analyzed as described above. Crude extract (27–64%) and the five compounds, SH (77–96%), DMAS (37–71%), AS (39–72%), IBS (61–89%), and IVS (25–58%), significantly inhibited TNF-α promoter activity in a dose-dependent manner as compared with the untreated control (Fig. 5). Among all the treatments, SH exhibited the highest inhibitory effect followed by IBS.

Shikonin and Isobutyryl Shikonin Suppress Transgenic Human TNF-α mRNA Expression and Protein Production in Vivo—As SH and IBS exhibited the highest inhibitory effects on TNF-α promoter activity, we further tested these two compounds *in vivo* for their effects on human TNF-α mRNA expression and protein production. To mimic physiological conditions, plasmid pTNFP-TNFC containing a human TNF-α cDNA driven by human TNF-α promoter was constructed and delivered into mouse skin via gene gun for *in vivo* transgenic expression of human TNF-α cytokine. Mouse skin transfected with pTNFP-TNFC was treated with SH, IBS, and hydrocortisone, each at a concentration of 100 μg/site/mouse. Skin samples were analyzed at 16 h post-transfection for human TNF-α mRNA expression by RT-PCR analysis. As shown in Fig. 6*A*, SH and IBS suppressed human TNF-α mRNA expression as compared with the untreated control, and these effects were stronger than that obtained with hydrocortisone. To determine the optimum time point for maximum production of human TNF-α cytokine in mouse skin, a time course experiment was performed. Skin tissue transfected with plasmid pTNFP-TNFC showed time-dependent production of TNF-α protein reaching a maximum level at 16 h post-transfection that was 83-fold higher than that of the control skin samples obtained at 0 h (Fig. 6*B*). Therefore, this time point was used for further analysis. To study the effect of the test compounds on human TNF-α protein production, plasmid pTNFP-TNFC-transfected skin was treated with each of SH, IBS, BP crude extract, and hydrocortisone at a concentration of 100 μg/site/mouse. SH and IBS treatments significantly suppressed the TNF-α protein production by 93 and 87% respectively as compared with the untreated control (Fig. 6*C*). Hydrocortisone also suppressed TNF-α protein production but only by 47%. However, crude extract of BP did not show any effect on TNF-α protein production in skin tissue.

Shikonin Does Not Inhibit the Phosphorylation of Erk1/2 and NF-κB p65 Induced by Particle-mediated Injury/Stress in Mouse Skin—The mitogen-activated protein kinases are known to mediate biological effects upon mechanical injury/stress. Physical stress has been shown to activate the Erk1/2 pathway. Injury and inflammation have been shown to activate the NF-κB pathway. To determine whether mitogen-activated protein kinases and/or NF-κB are activated in mouse skin in response to particle-mediated injury/stress, Western blot analyses with phospho-Erk1/2 and phospho-NF-κB p65 antibodies were carried out. Mouse skin was bombarded with pTNF-Luc DNA-coated gold particles, and samples were collected at different time points. Total protein extracts were subjected to Western blot analysis. Particle-mediated injury/stress resulted in a rapid increase in Erk1/2 phosphorylation within 30 min that began to decline after 1 h and remained unchanged for 4 h. Phosphorylation of NF-κB p65 was detected within 30 min of bombardment and continued to increase for up to 4 h afterward (Fig. 7*A*). To determine the effect of shikonin on the phosphorylation of Erk1/2 and NF-κB induced by particle-mediated injury/stress, mouse skin was bombarded with pTNF-Luc DNA-coated gold particles and was either untreated or treated with solvent alone or shikonin (100 μg/site/mouse). After 1 h, total protein was extracted from mouse skin samples and analyzed by Western blotting as described above. Shikonin treatment did not inhibit the phosphorylation of Erk1/2 or of NF-κB p65 (Fig. 7*B*).

Shikonin and Isobutyryl Shikonin Interfere with Human TNF-α Basal Transcription Machinery—Our results indicated that shikonins from LE inhibited the transcriptional activity of the human TNF-α promoter induced by particle-mediated physical injury/stress and thereby its mRNA expression and protein production. However, Western blot analysis revealed that shikonin did not inhibit the activation of Erk1/2 and NF-κB p65 induced by particle-mediated injury. We therefore extended our studies to identify the *cis*-regulatory elements of the TNF-α promoter that may mediate the suppressive effects of shikonin and isobutyryl shikonin. For this purpose, we generated a series of 5′-deletion mutants of different lengths relative to the transcription site from full-length (−1049/+48 bp)

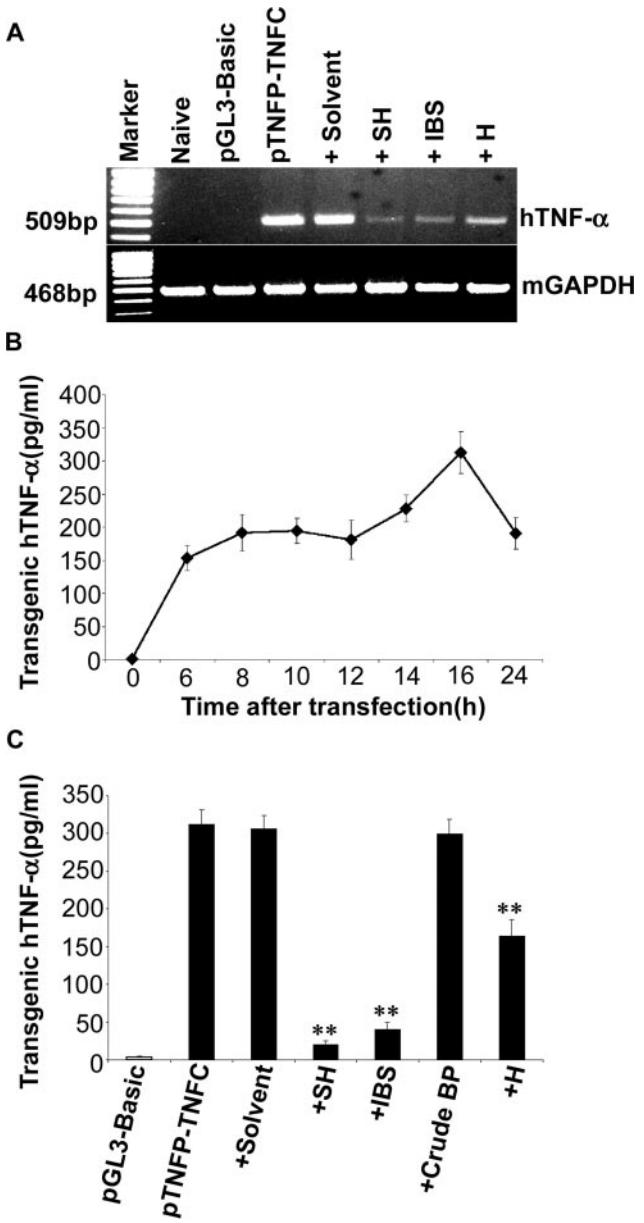

FIG. 6. **Shikonin and isobutyryl shikonin suppress transgenic human TNF-α mRNA and protein expression in mouse skin tissues.** *A*, mouse skin was untransfected or transfected with negative control vector pGL3-Basic or transgenic human TNF-α expression plasmid pTNFP-TNFC. pTNFP-TNFC-transfected skin was either untreated, solvent-treated, or treated with 100 μg each of SH, IBS, BP crude extract, and hydrocortisone (*H*)/site/mouse. Skin samples were collected 16 h post-transfection. Total RNA was extracted from tissues and analyzed by RT-PCR as described under "Experimental Procedures." Mouse GAPDH mRNA expression served as a control for RNA input. *B*, plasmid pTNFP-TNFC was transfected into mouse skin, and skin samples were collected at different time points, processed, and analyzed for transgenic human TNF-α protein production by using a specific ELISA kit as described under "Experimental Procedures." *C*, mouse skin was transfected with negative control vector pGL3-basic (□) or pTNFP-TNFC (■) that was either untreated, solvent-treated, or treated with 100 μg each of SH, IBS, BP crude extract, and hydrocortisone (*H*)/site/mouse. 16 h after treatment, skin samples were collected, processed, and analyzed as described above. The mean values obtained from three independent transfections of a representative experiment are shown (±S.D.). **, $p < 0.01$ *versus* untreated control. *h*, human; *m*, mouse.

human TNF-α promoter. These mutants were transfected into mouse skin by a gene gun transfection method. No significant differences were observed in promoter activity induced by gene gun-mediated physical injury/stress among the full-length promoter −1049/+48 bp and five deletion mutants −924/+48, −745/+48, −495/+48, −285/+48, and −124/+48 bp, whereas deletion of the promoter region to −29 bp relative to the transcription start site resulted in a 95% decrease but not complete abolishment of the promoter activity (Fig. 8). The −29 bp mutant, containing the TATA box and +48 bp downstream from the transcription start site without any upstream elements, still had a basal transcriptional activity that was 150-fold higher than that of the promoterless negative control vector (see Fig. 2). Mouse skin tissues transfected with full-length and six deletion mutants of human TNF-α promoter were treated with 100 μg of each of the test compounds. Shikonin and isobutyryl shikonin significantly inhibited promoter activity of all the deletion mutants, including the core promoter activity, by 84–99% as compared with the respective untreated controls (Fig. 8). These results indicated that the minimum promoter region sufficient for shikonins to exert their inhibitory effects was localized between −29 and +48 bp of TNF-α promoter.

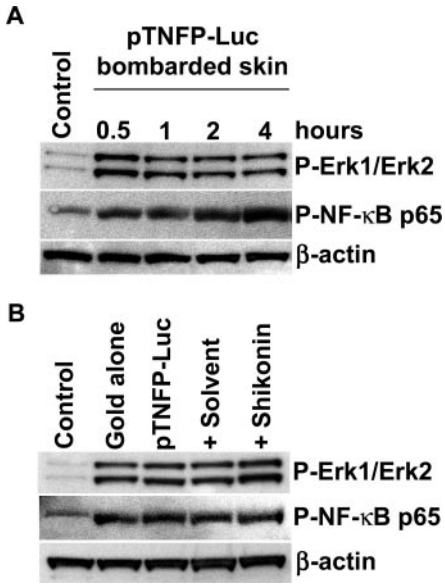

FIG. 7. **Shikonin does not inhibit the phosphorylation of Erk1/2 and NF-κB induced by particle-mediated injury/stress.** *A*, total protein extracts were prepared from pTNFP-Luc DNA-coated gold particle-bombarded skin samples at the indicated time points. Phosphorylation of Erk1/2 and NF-κB was detected by Western blot analysis using respective phospho-specific antibodies. *B*, pTNFP-Luc DNA-bombarded mouse skin was either untreated, treated with solvent, or treated with shikonin (100 μg/site/mouse). After 1 h, total protein extracts were prepared from skin samples and used for Western blot analysis as described above. Mouse β-actin served as a control for protein input. *P*-, phospho-.

Shikonin Inhibits the Binding of TFIID Complex (TATA Box-binding Protein (TBP)) to TATA Box in TNF-α Promoter—Since TATA box is a fundamental element in the core promoter responsible for the assembly and initiation of transcription and TFIID is the first basal transcription factor assembled onto promoters through binding of TBP to the TATA box, we conducted EMSA to determine whether shikonin suppresses the binding of TFIID complex (TBP) to TATA box. To mimic the experimental conditions previously used for transgenic promoter inhibition, mouse skin was bombarded with pTNFP-Luc DNA-coated gold particles and was either untreated or treated with solvent alone or shikonin at concentrations of 1, 5, 10, 50. and 100 μg/site/mouse. Skin samples were harvested after 1 h, and nuclear extracts were subjected to EMSA by using the −30 bp core promoter region of human TNF-α as a DNA probe that contains the only DNA binding element, TATA box. In untreated and solvent-treated samples, TFIID complex binding

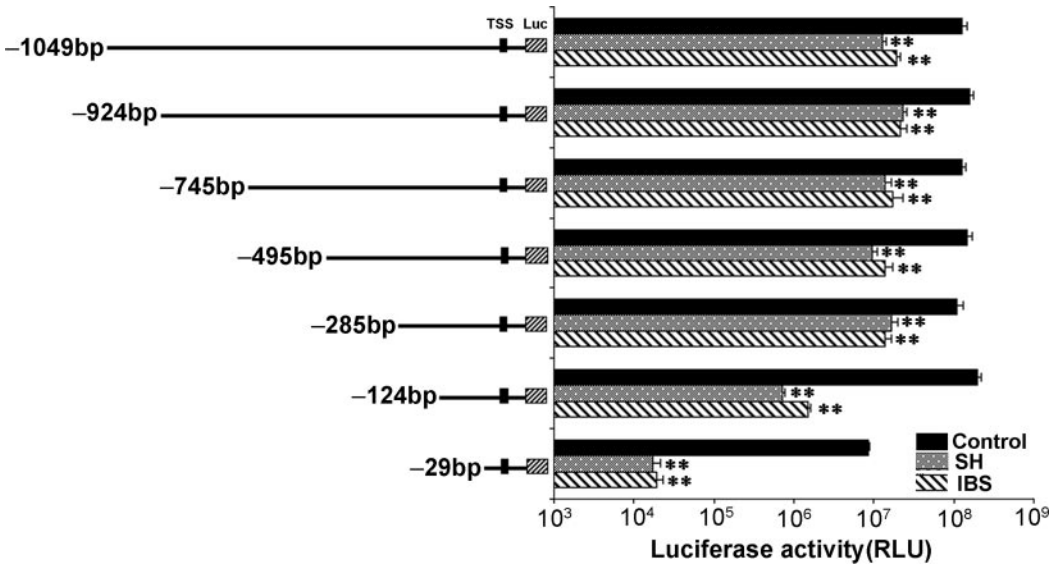

FIG. 8. **The minimal human TNF-α promoter region responsive to the suppressive effects of shikonin and isobutyryl shikonin.** Mouse skin was transfected with plasmids containing full-length (−1049/+48 bp) or a series of 5′-deletion mutants of TNF-α promoter linked to a luciferase reporter gene at a dose of 2.5 μg/site/mouse by gene gun delivery. Skin transfected with each deletion mutant was untreated or treated with 100 μg each of shikonin and isobutyryl shikonin. Untreated samples were used as controls. Skin samples were harvested and processed as described in Fig. 2. The mean values obtained from three independent transfections of a representative experiment are shown (±S.D.). **, $p < 0.01$ *versus* respective untreated control. *RLU*, relative light units; TSS, transcription start site.

was clearly detectable (Fig. 9, *lanes 2* and *4*), whereas in shikonin-treated samples the binding of TFIID complex was inhibited in a dose-dependent manner (*lanes 5–9*). Specificity of binding was indicated by prevention of the signal shift by competition from a 200-fold excess of unlabeled DNA probe (*lane 3*). To determine whether direct binding of shikonin to the TATA box prevented the TBP binding, we performed EMSA with the binding reactions containing no nuclear extract but the labeled probe and shikonin at concentrations of 0.01, 0.1, 0.5, 1, and 5 μg. Shikonin binding was not observed in any of the reactions (*lanes 10–14*). These results indicate that shikonin itself does not bind to the TATA box but may affect the binding of TFIID complex (TBP) either directly or indirectly.

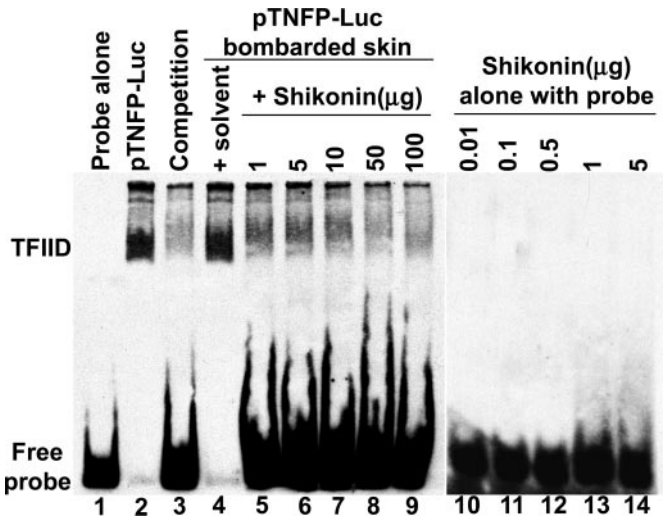

FIG. 9. **Shikonin inhibits the TFIID complex (TBP) binding to TATA box in TNF-α promoter.** Mouse skin was bombarded with pTNF-Luc DNA-coated gold particles and either untreated or treated with solvent alone or shikonin at different concentrations. Skin samples were harvested after 1 h, and nuclear extracts were subjected to EMSA as described under "Experimental Procedures" (*lanes 2* and *4–9*). Binding specificity of the complexes was analyzed with 200-fold excess of unlabeled DNA probe competition (*lane 3*). Direct binding of shikonin to the probe was tested by incubating different concentrations of shikonin with the labeled probe (*lanes 10–14*).

DISCUSSION

Molecular studies of the physiology of the inflammatory response have identified a hierarchy of cytokine activities. TNF-α seems to be the cytokine at the apex of the proinflammatory cytokine cascade, and its production is the primary event that initiates and orchestrates the inflammatory response (27). Since many of the effects of excessive TNF-α are deleterious to the organism, it is of great interest to find ways of blocking its production and its action on host cells. Previous efforts in this area have focused on the use of agents that inhibit TNF-α production (28), inactivation of TNF-α by the use of monoclonal antibodies (29), antibodies to block TNF-α receptors (30), and soluble derivatives of the two TNF-α receptors (31). However, most of these efforts have been directed at inhibiting the undesirable effects of the cytokine, which has already been produced and is present in the organ, tissue, cell, and receptor systems of the body. Here we have used a new *in vivo* approach that utilizes the inhibition of TNF-α promoter-driven luciferase-reporter gene expression as a target for identifying the novel anti-inflammatory agents.

Particle bombardment by gene gun technology is an efficient physical method of gene transfer in which high density, subcellular sized gold particles are accelerated to high velocity to carry DNA into cells. In the current study, we observed that the microscopic tissue injury and stress caused by gene gun particle bombardment resulted in localized cutaneous inflammatory response (erythema) (Fig. 3*A*) and a relatively high level of human TNF-α promoter activity in mouse skin tissue. We hypothesized that the particle bombardment-mediated physical injury/stress may have induced the transgenic human TNF-α promoter activation. This was confirmed through the induction of endogenous mouse TNF-α mRNA expression and protein production by particle-mediated physical injury/stress (Fig. 3, *B* and *C*). Recent studies have shown that mechanical stress can result in activation of distinct mitogen-activated protein kinase signaling pathways in skeletal muscle fibers (32), and inflammation and injury have been reported to activate the NF-κB signal transduction pathway in keratinocytes (33). In our study we found that gene gun particle-mediated injury/stress induced the activation of Erk1/2 and nuclear fac-

tor-κB (Fig. 7*A*). Previous studies have reported the involvement of mitogen-activated protein kinase signal transduction pathways and the NF-κB pathway in the induction of TNF-α by glass fibers in rat alveolar macrophages (34). The direct association of the phosphorylation of Erk1/2 and NF-κB with TNF-α promoter activation in mouse skin was not determined in the present study. However, the 5′-deletion analysis of the human TNF-α promoter *in vivo* revealed that the minimal promoter region responsive to particle-mediated physical injury/stress induction of TNF-α transcription is −124/+48 bp relative to the transcription start site, and deletion of this region to −29/+48 bp significantly decreased the promoter activity (Fig. 8). This region contains putative consensus binding sites for potential transcription factors such as Ets, cAMP-response element, k3, nuclear factor of activated T cell, AP-1, SP-1, and AP-2 (35). Transcription of TNF-α is controlled by multiple regulatory elements such as the k3 site (NF-κB binding site), cAMP-response element, and AP-1 binding site. Upon activation, NF-κB translocates to the nucleus where it binds specific sites in the promoter regions of responsive genes. Activation of Erk1/2 in turn results in the phosphorylation and activation of the transcription factor cyclic AMP-response element-binding protein and the ETS domain transcription factor (Elk1) (36). It has been reported that both the k3 site and cAMP-response element are required for a maximal induction of TNF-α transcription, and a synergy between these two elements is necessary (37). Therefore, we speculate that either Erk1/2 or NF-κB or both pathways may be involved in the induction of TNF-α promoter activation in response to physical injury/stress in mouse skin. To our knowledge, this is the first report to show that human TNF-α promoter can be activated by physical injury/stress *in vivo* in skin tissue, although previous *in vitro* studies have shown that mechanical stress induces the production of TNF-α in rat cardiac fibroblasts and myocardium (38, 39). Therefore, we propose from this study that particle-mediated physical injury/stress-induced TNF-α promoter activity can be used efficiently for evaluating the effects of anti-inflammatory agents.

In the current study, we evaluated the anti-inflammatory effects of shikonin derivatives isolated from *L. erythrorhizon* by targeting the important proinflammatory cytokine TNF-α. Our results, based on reporter gene activity in an *in vivo* mouse skin system, show that a crude extract of *L. erythrorhizon* significantly inhibited the physical injury/stress-induced transcriptional activation of human TNF-α promoter, and the level of inhibition was comparable to that of the commercially available topical anti-inflammatory corticosteroids hydrocortisone and betamethasone (Fig. 4). The five tested shikonins, SH, IBS, DMAS, AS, and IVS, significantly inhibited the transcriptional activation of human TNF-α promoter in a dose-dependent manner. Each compound differs from shikonin by a single R group moiety. Based on structure and activity relationship analysis, we suggest that shikonin derivatives differing in substitution at the C-1′ position (Fig. 1) may confer a different effect on the TNF-α promoter activity. The strongest inhibition was observed when a hydroxyl group is located at the C-1′ position, *i.e.* as seen for shikonin compound (96% inhibition at 200 μg/site) (Fig. 5). Moreover replacing the hydroxyl group with an ester linkage at the C-1′ position resulted in significant but varying levels of decrease in the inhibition of TNF-α promoter activity. The ranking of inhibitory activity of the shikonin derivatives with esteryl group substitutes is isobutyl group (IBS), acetyl group (AS) = dimethylacryl group (DMAS), and isovaleryl group (IVS) with levels of inhibition ranging from 58 to 89% at the dose of 200 μg/site (Fig. 5). These results indicate that each shikonin compound has the same biological effect, although the intensity of the effect varied from compound to compound, which may directly correlate to the moiety of the R group located at the C-1′ position. In addition to these *in vivo* studies, our *in vitro* data also indicate that shikonin can inhibit the lipopolysaccharide-induced transgenic human TNF-α promoter activity in THP1, a human monocytic cell line (data not shown). This suggests that irrespective of the system (*in vivo* or *in vitro*) and the inducer (physical injury/stress or lipopolysaccharide) used, shikonin is able to suppress transgenic human TNF-α promoter activity. It has been shown that the suppression of TNF-α transcription and its secretion by classical anti-inflammatory drugs, such as salicylates and glucocorticoids, contribute to their efficacy in the treatment of some inflammatory diseases (40, 41). We have demonstrated here that shikonin and isobutyryl shikonin not only inhibited promoter activity but also suppressed transgenic human TNF-α mRNA expression and protein production in mouse skin tissue (Fig. 6), indicating the potential application of these phytocompounds as anti-inflammatory therapeutics.

Our experiments to elucidate the molecular mechanism underlying the inhibitory effects of shikonin compounds on TNF-α promoter activation demonstrate that shikonin does not inhibit the phosphorylation of Erk1/2 and NF-κB induced by particle bombardment-mediated physical injury/stress, indicating the involvement of an alternative mechanism (Fig. 7*B*). Through functional analysis using a series of promoter deletion constructs, we have shown that the inhibitory effect of shikonin is localized to a core promoter region containing the TATA box and +48 bp of the TNF-α promoter (Fig. 8). These results suggest that shikonin interferes with the basal transcription machinery of TNF-α promoter and abolishes the core promoter activity to the background level. Transcription initiation by RNA polymerase II involves the assembly of general components of basal transcription machinery, such as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (42). TFIID is a multisubunit complex consisting of a DNA-binding subunit, the TBP, and a set of TBP-associated factors. TFIID is the first basal transcription factor assembled onto RNA polymerase II-dependent promoters through binding of TBP to the TATA box (43). TBP has been reported as the only subunit of TFIID required for basal transcription *in vitro* (44). In this study, we have found that shikonin inhibits the binding of TFIID complex (TBP) to TATA box in TNF-α promoter and thereby suppresses its basal transcription as well as activator-regulated transcription (Fig. 9). Our data excluded the possibility of shikonin directly binding to TATA box thereby preventing the TBP (TFIID) binding. Previous mechanistic studies of shikonin indicated that it is an effective inhibitor of protein-protein interactions with multiple targets in both intracellular and extracellular compartments (45). In the present investigation we propose that the inhibitory effect of shikonin on binding of TFIID complex (TBP) to TATA box could be due to the inactivation of TBP either by directly altering the association of TBP with TBP-associated factor subunits within TFIID or indirectly through host mechanisms that are capable of inhibiting the TBP activity. An alternative possibility is that shikonin may directly or indirectly interact with the proteins that inhibit the transcription factor binding to DNA by remodeling and modifing the structure of chromatin. Further investigation is required to clarify the shikonin inhibitory effect on binding of TFIID complex (TBP) to TATA box.

In summary, this study demonstrates for the first time a new approach for quantitative molecular screening of anti-inflammatory agents by using an *in vivo* skin system that reflects the physiological conditions. Our results suggest that shikonins from *L. erythrorhizon* plant suppress the transcriptional activation of the TNF-α promoter by inhibiting the binding of

TFIID complex (TBP) to TATA box within the basal transcription machinery and thereby the subsequent expression of the TNF-α protein. Thus, shikonins may have important therapeutic benefits for skin-related inflammatory disorders and also possibly for systemic inflammatory disease conditions associated with increased TNF-α production. Further studies to determine whether shikonin and its derivatives also suppress the expression of other proinflammatory and immunoregulatory cytokine genes would provide greater insight into their potential therapeutic use as anti-inflammatory and immunosuppressive agents.

Acknowledgments—We thank Yi-Jie Chen, Shih-Hsiang Hsiao, Toyota, and Ming-Tsang for technical help in carrying out this research. We thank Dr. Kenrick Deen for editing the manuscript.

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