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ARTICLE

GMI, a *Ganoderma* Immunomodulatory Protein, Down-regulates Tumor Necrosis Factor α-Induced Expression of Matrix Metalloproteinase 9 via NF-κB Pathway in Human Alveolar Epithelial A549 Cells

Ching-Hsiung Lin,[†,§] Yi-Min Hsiao,[#,∥] Chu-Chyn Ou,[○] Yu-Wen Lin,[⊥,∥] Yi-Lin Chiu,[⊗,●] Ko-Huang Lue,[△] Jan-Gowth Chang,*[,§,◆] and Jiunn-Liang Ko*[,⊥,△]

[†]Division of Chest Medicine, Changhua Christian Hospital, Changhua, Taiwan, Republic of China, [§]Graduate Institute of Medicine, Kaohsiung Medical University, Taiwan, Republic of China, [#]Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Taichung, Taiwan, Republic of China, [⊥]Institute of Medicine, Chung-Shan Medical University, Taichung, Taiwan, Republic of China, [⊗]Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan, Republic of China, [●]Taichung Branch of Armed Forces Medical Supplies Office, Taichung Armed Forces General Hospital, Taichung, Taiwan, Republic of China, [△]Division of Allergy, Department of Pediatrics, Chung-Shan Medical University Hospital, Taichung, Taiwan, Republic of China, [◆]Department of Laboratory Medicine, Kaohsiung Medical University Hospital, 100 First Road, Kaohsiung, Taiwan, Republic of China, and [○]Department of Nutrition, Chung-Shan Medical University, Taichung, Taiwan, Republic of China. [∥]Both authors equally contributed to this work as second authors.

Matrix metalloproteinase 9 (MMP-9) has been implicated in airway injury in chronic obstructive pulmonary disease (COPD), lung inflammation, and lung cancer and plays a major role in tumor necrosis factor-α (TNF-α)-stimulated tumor invasion and lung inflammation. MMP-9 activity is promoted by the pro-inflammatory cytokine TNF-α. GMI, cloned from *Ganoderma microsporum* and purified, is one of the recombinant fungal immunomodulatory proteins. To understand the molecular mechanisms involved in the suppression of TNF-α-mediated tumor invasion and inflammation, GMI modulation of this pathway was investigated in human alveolar epithelial A549 cells in this study. GMI exhibited an inhibitory effect on TNF-α-induced invasion, with GMI treatment and TNF-α exposure presenting the most anti-invasive properties on Boyden chamber assay. GMI reduced TNF-α-induced MMP-9 activities on gelatin zymography assay through inhibition of MMP-9 transcriptional activity. RT-PCR and MMP-9 promoter luciferase analysis revealed that GMI inhibits the transcription of MMP-9 mRNA. Moreover, in vitro and in vivo binding experiments, an electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation assay (ChIP) demonstrated that GMI suppresses DNA binding of nuclear factor (NF)-κB transcription factors to MMP-9 promoter. Western blot analysis indicated that GMI blocks the phosphorylation and degradation of IκBα, which in turn leads to suppression of the phosphorylation and nuclear translocation of p65. Thus, overall, our results indicated that GMI mediates antitumor invasion and anti-inflammatory effects through modulation of NF-κB/MMP-9 pathways.

KEYWORDS: Immunomodulatory protein; GMI; tumor necrosis factor-α; NF-κB; MMP-9; invasion

INTRODUCTION

Airway remodeling is a provital feature of the pathogenesis of chronic asthma, chronic obstructive pulmonary disease (COPD), and inflammation. Irreversible structural changes such as subepithelial collagen deposition, goblet cell hyperplasia, and epithelial desquamation are observed in these diseases, indicating that epithelial and alveolar cells are important targets for airway remodeling. Matrix metalloproteinases (MMPs) are responsible for degradation of the extracellular matrix (ECM) components, including basement membrane collagen, interstitial collagen, fibronectin, and various proteoglycans, during normal remodeling and repair processes (*1*). The principles of MMP biology focus on the clinical and experimental evidence related to MMP activity in various lung disorders, including lung cancer, pleural effusions,

*Address correspondence to both authors [(J.-L. K. and J.-G. C) Institute of Medicine, Chung-Shan Medical University, 110 Sec. 1, Chien-Kuo N. Road, Taichung, Taiwan 40203, Republic of China (phone) (886-4) 24730022-11694, (fax) (886-4) 24751101, (e-mail) jlko@csmu.edu.tw; (J.-G.C.) (e-mail) jgchang@ms.kmuh.org.tw].

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asthma, and COPD (*2*). Furthermore, the epithelium is the primary target for environmental inflammatory agents, such as allergens and house dust, and is involved in proinflammatory mediator recruitment. Development of a novel pharmacological drug directed against lung inflammation is of the highest priority.

Lingzhi (a species of Basidiomycetes), *Ganoderma lucidum*, is a traditional medicine. Although in use for thousands of years, it lacks an established molecular basis as defined within the past half-century. In recent years, many therapeutic effects have been reported for Lingzhi species, such as inhibition of proliferation, anti-inflammation, antitumor promotion, and immunomodulatory effects (*3*). Lingzhi contains a wide variety of bioactive molecules, including polysaccharides, glycoproteins, and triterpenoids (*4*). A new glycoprotein class in Lingzhi has been identified and named fungal immunomodulatory proteins (FIPs). So far, six FIPs have been isolated and purified, including four from Lingzhi, LZ-8 (*G. lucidum*), FIP-gts (*Ganoderma tsugae*), FIP-GMI (*Ganoderma microsporum*), and FIP-gja (*Ganoderma japonicum*), and two from edible mushrooms, FIP-fve (*Flammulina velutipes*) and FIP-vvo (*Volvariella volvacea*) (*5*). From our previous study, recombinant FIP-*gts* suppresses telomerase activity and inhibits transcriptional regulation of human telomerase reverse transcriptase (hTERT) via a c-myc-responsive element-dependent mechanism (*6*). In addition, reFIP-*gts*-treated lung cancer cells are arrested at G1 phase by flow cytometry and possess a morphological phenotype consistent with premature cellular senescence (*7*). In an in vivo mouse model, FIP-*gts* results in significant inhibition of tumor growth in athymic nude mice implanted with A549 cells (*7*). An immunomodulatory protein, Ling Zhi-8 (LZ-8), enhances IL-2 expression within human T cells via src-family protein tyrosine kinase (*8*). In this study, GMI was cloned from *G. microsporum*, and the amino acid sequences of GMI and FIP-*gts* were found to be highly homologous with those of the other FIPs. GMI contains 111 amino acids, which are homologous with 83.8% of the protein sequence between GMI and FIP-gts. Our previous study indicated that GMI inhibits epidermal growth factor (EGF) mediated migration and invasion in A549 (*9*).

Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent proteases that digest most extracellular matrix components, such as collagens, fibronectins, and proteoglycans. The secreted form of MMPs is latent proenzymes, and the proteolytic cleavage of an amino-terminal domain converts MMPs to the active form. In general, the levels of MMPs are very low. MMPs are induced by stimulated factors such as IL-1, TNF-α, EGF, and TGF-β (*10*, *11*). Matrix metalloproteinase-9 (MMP-9) (gelatinase B, 92 kDa) plays an important role in cell development, connective remodeling, wound healing, angiogenesis, tumor invasion, immunity, inflammation, and metastasis. Elevated expression of MMP-9 is related to increased metastatic potential in several cancer types including breast cancer, pancreatic cancer, ovarian cancer, prostate cancer, and lung cancer (*12*, *13*).

TNF-α is one type of proinflammatory cytokine that promotes carcinoma progression through stimulation of malignant epithelial cell migration, invasion, and metastasis (*14*). MMP-9 is a TNF-α-responsive extracellular matrix-degrading enzyme. By degrading basement membrane, MMP-9 promotes carcinoma cell migration and invasion. TNF-α stimulates MMP-9 expression in a nuclear factor κB-dependent manner, as the promoter of MMP-9 gene contains binding sites for nuclear factor-κB (NF-κB), contributing to TNF-α induced malignant cell invasion and migration (*15*).

In this work, we evaluated the effects of GMI, which has been shown to inhibit cell metastasis of TNF-α induced invasion, in A549 lung cancer cells. Focusing on mechanisms for the inhibition of invasion mediated by GMI, we showed that GMI suppresses MMP-9 activity and expression by reduced MMP-9 transcriptional activity. Furthermore, we demonstrated that the down-regulation of MMP-9 results from decreased translocation of NF-κB to the nucleus and increased phosphorylation, ubiquitination, and degradation of IκBα by TNF-α stimulation. Thus, GMI may be a potential chemopreventive agent against invasive lung tumors.

MATERIALS AND METHODS

Reagents. Bacteria-derived human recombinant tumor necrosis factor (TNF-α), purified to homogeneity with a specific activity of $\geq 2 \times 10^7$ units/mg, was provided by Peprotech Inc. (Rocky Hill, NJ). Penicillin, streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was supplied by Gibco (Carlsbad, CA). Antibodies against NF-κB p65 were obtained from Millipore (Bedford, MA) for Western blot and immunocytochemistry analyses. Anti-IκBα phosphospecific anti-IκBα (Ser32/Ser36) and phos−phospecific anti-NF-κB p65 (Ser536) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Other antibodies included monoclonal anti-β-actin antibody (Sigma, St. Louis, MO) and Sp1 antibody (Upstate Biotechnology, Lake Placid, NY).

Expression of GMI Protein. GMI is highly homologous with FIP-*gts*. GMI, manufactured by Mycomagic Biotechnology Co., Ltd. (Taipei, Taiwan), was generated and ameliorated from *G. microsporum* (*16*). GMI was cloned and purification methods were as described previously (*9*). The endotoxin levels of GMI were determined by *Limulus* amebocyte lysate assays (< 1.0 EU/μg).

Cell Lines. The human lung carcinoma cell line A549 was obtained from American Type Culture Collection (ATCC; CCL-185). A549 cells were cultured in DMEM supplemented with 10% FBS. All culture media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

MMP-9 Gelatin Zymogram. A549 cells were plated onto 24-well culture plates and made quiescent at confluence by incubation in serum-free DMEM for 24 h. Growth-arrested cells were incubated with or without different concentrations of TNF-α at 37 °C for the indicated time periods. When inhibitors were used, they were added 1 h prior to the application of TNF-α. After treatment, the culture medium was collected and centrifuged at 14000 rpm at 4 °C for 5 min to remove cell debris. The culture medium was mixed with 6× nonreducing sample buffer (5:1, v/v) and electrophoresed on 8% SDS-PAGE containing 0.1% gelatin as an MMP substrate. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 at room temperature for 30 min to remove SDS. The gel was then incubated in reaction buffer [400 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, and 0.01% NaN_3] at 37 °C overnight and stained with Coomassie brilliant blue R-250.

Total RNA Extraction and RT-PCR Analysis. Total RNA was isolated from A549 cells in 10 cm culture dishes treated with TNF-α in the presence of RareRNA (Genepure Technology, Taiwan) for the indicated times, according to the protocol of the manufacturer. RNA concentration was spectrophotometrically determined at 260 nm. First-strand cDNA synthesis was performed with 2 μg of total RNA using random hexamers as primers in a final volume of 20 μL (100 ng/μL random hexamers, 1 mM dNTPs, 1 U/μL RNasin, and 5 U/μL Moloney Murine Leukemia Virus Reverse Transcriptase). The reaction was carried out at 42 °C for 90 min (*17*). cDNAs encoding β-actin and MMP-9 were amplified from 1 μL of the cDNA reaction mixture using specific gene primers. Oligonucleotide primers for β-actin and MMP-9 were as follows: β-actin, 5′-TCATCACCATTGGCAATGAG-3′ (sense), 5′-CACTGTGTTGGCGTACAGGT-3′ (antisense); MMP-9, 5′-CAACATCACCTATTGGATCC-3′ (sense), 5′- CGGGTGTAGAGTCTCTCGCT-3′ (antisense). DNA amplification was achieved by annealing at 50 °C for MMP-9 and at 55 °C for β-actin for 1 min each. MMP-9 and β-actin sequences were amplified for 38 and 28 cycles, respectively. These primer sets specifically recognized the genes of interest as indicated by amplification of single bands of the expected sizes (480 and 154 bp for MMP-9 and β-actin, respectively). The PCR products were analyzed on 1.5% agarose 0.5× TBE gel containing ethidium bromide, and their sizes were compared to molecular weight markers.

Measurement of MMP-9 Promoter Activity. A 710 bp (−720 to −11) segment from the 5′-promoter region of the MMP-9 gene was cloned

as described previously (*18*). Briefly, a 0.71 kb segment of the 5′-flanking region of the human MMP-9 gene was amplified by PCR using specific primers with restriction enzyme site from the human MMP-9 gene (accession no. D10051): 5′-ACATTTGCCCGAGCTCCTGAAG (forward/*Kpn*I) and 5′-AGGGGCTGCCAGAAGCTTATGGT (reverse/*Hin*dIII). The pGL3-Basic vector, containing a polyadenylation signal upstream from the luciferase gene, was used to construct the expression vectors by addition of PCR-amplified DNA with restriction enzyme site of the MMP-9 promoter to the *Kpn*I/*Hin*dIII site of this vector. The PCR products (pGL3-MMP-9) were confirmed by size, as determined by electrophoresis and DNA sequencing. pGL3-MMP-9 plasmid was transfected into A549 cells using the jetPEI reagent (Polyplus- transfection) according to the manufacturer's instructions. After incubation with TNF-α (10 ng/mL), cells were collected and disrupted by Luciferase Assay System (Promega). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the Luciferase Assay System. Firefly luciferase activities were standardized for β-galactosidase activity.

Cell Invasion Assay. Cell invasion assays were performed using modified Boyden chambers 6.5 mm in diameter, with 10 mm thick porous (8 μm) polycarbonate membrane separating the two chambers (Transwell; Costar, Cambridge, MA). The membrane of the upper chamber was coated with Matrigel (3 μg/well; BD Biosciences Discovery Labware) for 3 h. Condition medium was prepared from A549 cells, which were pretreated with or without GMI for 8 h. To the medium of the bottom chamber was added 10% FBS−DMEM containing TNF-α (10 ng/mL) or condition medium. Cells were trypsinized, centrifuged, and resuspended at 4×10^5 cells/mL in 0.5% FBS−DMEM. After 24 h of incubation, the cells on the upper well and the membranes coated with Matrigel were fixed with methanol and stained with 20% Giemsa solution (Merck). The cells that were attached to the lower surface of the polycarbonate filter were counted under a light microscope (magnification, ×100). The experiments were performed in triplicate.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). A nuclear extract was prepared. Briefly, cultured cells were collected by scraping in HED buffer [containing 25 mM HEPES (pH 7.6), 1.5 mM EDTA, 1 mM DTT, 1× protease inhibitor (Roche), and 0.5 mM PMSF] with 10% glycerol. They were then spun down at 1800 rpm for 5 min. After 5 min, the cells were homogenized in HED buffer using a Wheaton Dounce Tissue Grinder Homogenizer and spun down at 7000 rpm for 10 min. The nuclear pellet was collected by centrifugation and extracted in HED buffer with 10% glycerol and 0.5 M NaCl at 4 °C for 1 h. The nuclear extract (10 μg) was incubated at 25 °C for 10 min with a 10 pmol biotin-labeled oligonucleotide spanning the −79 MMP-9 cis-element of NF-κB binding site. The sequence was as follows: NF-kB, CAGTGGAATTCCCCAGCC. The reaction mixture was electrophoresed at 4 °C in 6% polyacrylamide gel using TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) running buffer. The detecting solution was from Pierce Co., and the protocols were run according to the instructions of the manufacturer.

Chromatin Immunoprecipitation (ChIP) Assay. A549 cells were cultured in complete medium until 80% confluence (2×10^6/100 mm dish), changed to serum-free medium, and incubated overnight. When inhibitors were used, they were added 1 h prior to the application of TNF-α. The ChIP assay was carried out following the manufacturer's protocol (chromatin immunoprecipitation assay kit, catalog no. 17-295, Upstate Biotechnology Inc., Lake Placid, NY). Immune complexes were prepared using anti-NF-κB p65 antibody. The supernatant of an immunoprecipitation reaction carried out in the absence of antibody was used as the total input DNA control. PCR was carried out with 10 μL of each sample using the following primers: MMP-9 promoter-S, AGTGGTAAGACATTTGCCCGA; MMP-9 promoter-AS, TGCAACACCCCCTCCCAGGTCAG. This was followed by analysis on 2% agarose gels. Primers from the MMP-9 open reading frame that would amplify a 295 bp fragment were used as the PCR control.

Western Blot Analysis. A549 cells were cultured in complete medium until 80% confluence (2×10^6/100 mm dish) and then changed to serum-free medium and incubated overnight. A nuclear extract was prepared. Briefly, cultured cells were collected by centrifugation, washed, and suspended in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet NP-40. The nuclear pellet was then collected by centrifugation and extracted in buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF at 4 °C for 15 min. Extracts were prepared and fractionated by SDS−polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes, blotted with the relevant antibody, and detected by ECL reagent (Perkin-Elmer Life Sciences).

Immunocytochemical Analysis for NF-κB p65 Localization. The effect of GMI on TNF-α-induced nuclear translocation of p65 was examined using an immunocytochemical method. In brief, A549 cells were initially seeded onto 24-well plates with a coverslip. The cells were treated with GMI for 8 h followed by stimulation with 10 ng/mL TNF-α for 20 min. After treatment, slides were washed twice with PBS and fixed in a 3.7% paraformaldehyde−PBS solution at room temperature for 10 min. After two additional washes with PBS, cells were permeabilized with a solution of 0.1% Triton X-100 in PBS for 3−5 min and washed again with PBS. The slides were subsequently blocked with blocking solution (1% bovine serum albumin and 0.025% saponin in PBS) for 30 min and then incubated with rabbit polyclonal anti-NF-κB p65 antibody at 1:100 dilution followed by overnight incubation at 4 °C. The slides were again washed in PBS and incubated with goat anti-rabbit IgG−Texas Red at 1:100 dilution at room temperature for 1 h. After three washes with PBS, coverslips were mounted on a microscope slide with Prolong Gold antifade reagent with DAPI (Life Technologies). Imaging was performed with a confocal laser scanning microscope (Zeiss LSM510 BETA) at ×400 magnification.

Statistical Analysis. Results are reported as means ± SD. Statistical analysis was carried out using Bonferroni/Dunn test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

It has been suggested that the proteolysis of MMPs is involved in the processes of cell proliferation, migration, and tissue remodeling that are associated with the pathogenesis of airway inflammation. COPD, a global public health problem, is characterized by progressive difficulty in breathing, with increased mucin production, especially in the small airways. MMP-9 is thought to play important roles in mucin production by acrolein, a constituent of cigarette smoke and an endogenous mediator of oxidative stress (*19*). The basal level of MMP-9 is usually low, and its expression can be induced by TNF-α. The present study was designed to investigate the effect of GMI on the TNF-α-induced NF-κB activation pathway and on the regulation of MMP-9 to control epithelial cell invasion. We found that GMI suppressed the invasiveness of A549 cells with or without TNF-α treatment. In a previous study, MMP-9 promoted carcinoma cell migration and invasion. In this study, we found that GMI inhibits cell invasion through reduced MMP-9 transcription activity. This suppression of MMP-9 transcription activity by GMI was due to reduced activation of NF-κB binding to MMP-9 promoter.

GMI Inhibits Invasion of TNF-α-Stimulated A549 Cells. We investigated the effects of GMI on TNF-α-stimulated cell invasive ability and used a modified Boyden chamber assay to quantify the invasive potential of A549 cells. The results showed that GMI induces a dose-dependent decrease in invasion (**Figure 1A**). At 4 μg/mL, the proportion of invading cells was reduced to 69%, and at 16 μg/mL the proportion of invading cells was reduced to < 31%. Subsequently, GMI induced a dose-dependent decrease in TNF-α-stimulated invasion (**Figure 1A**). The TNF-α-induced invading A549 cells increased to 1.44 times the control levels with 10 ng/mL TNF-αtreatment and to 1.68 times the control levels with 100 ng/mL TNF-α treatment. In A549 cells treated with 10 ng/mL TNF-α, the addition of 4 and 16 μg/mL GMI led to decreases to 0.6 times the control levels (invading cell ratio decreased from 1.44 times to 0.87 times) and 0.32 times the control levels (invading cell ratio decreased from 1.44 times to 0.46 times), respectively. Ratios were similar to those in TNF-α-untreated

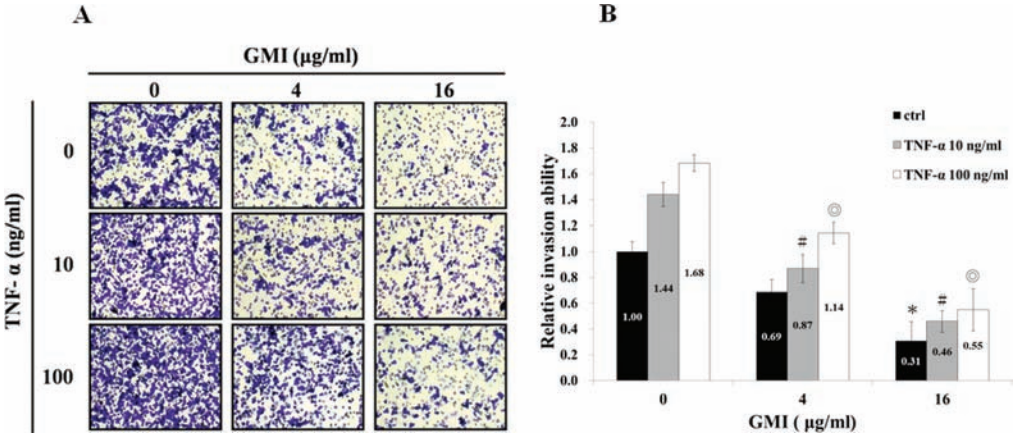

Figure 1. GMI inhibits TNF-α-induced invasion in A549 cells. A549 cells (2×10^4 cells/well) were seeded onto the upper chamber of membrane and treated with different concentrations of GMI (4, 16 μg/mL) for 8 h. The bottom chamber was filled with DMEM supplemented with 10 or 100 ng/mL TNF-α. The upper chambers of membrane were coated with 3 μg of Matrigel. After about 24 h, the invading A549 cells passed through the membrane and were quantified by counting the cells that migrated onto the membrane. Cells were fixed, stained, and counted as described in the text. Quantitative results are shown in panel **B**. The data are presented as mean ± SD of triplicate experiments. ∗, $p < 0.05$ versus untreated control; #, $p < 0.05$ versus TNF-α-treated (10 ng/mL); ◎, $p < 0.05$ versus TNF-α-treated (100 ng/mL).

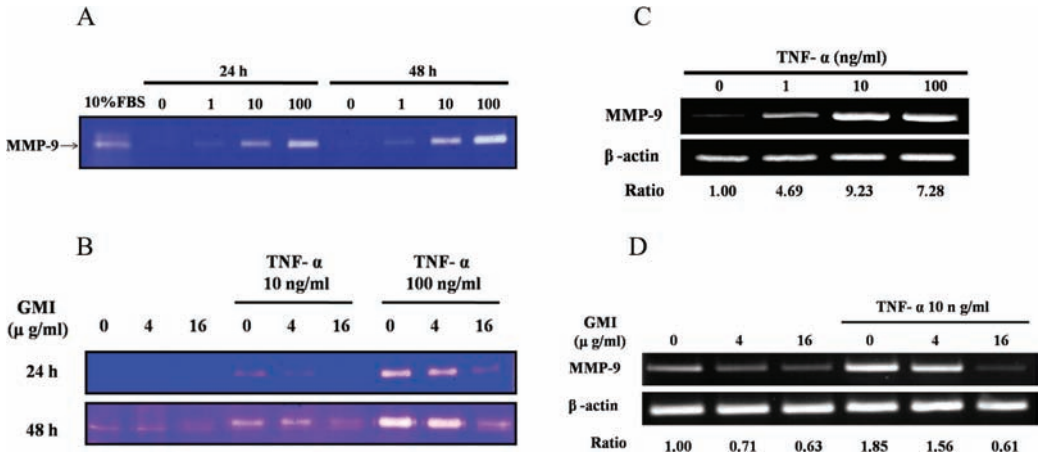

Figure 2. TNF-α induces MMP-9 expression and GMI inhibits TNF-α-induced MMP-9 expression in A549 cells. (**A**) Cells were seeded at 1.2×10^5 per 24 wells, cultured in serum-free medium at 37 °C for 24 h, and then treated with various concentrations of TNF-α for 24 and 48 h. Conditioned media were collected and analyzed by gelatin zymography. (**B**) A549 cells were pretreated with various concentrations of GMI (0, 4, 16 μg/mL). Cells were incubated 1 h later with 10 or 100 ng/mL of TNF-α for 23 and 47 h. For the zymography assay, the media were subjected to gelatin SDS-PAGE. (**C**) TNF-α-induced MMP-9 mRNA expression was concentration dependent. Cells were grown to confluence on 6 cm dishes (8×10^5 per 60 mm dish) and incubated with various concentrations of TNF-α (ng/mL) for 24 h. The isolated RNA samples were analyzed by RT-PCR, using the primers specific for MMP-9 and β-actin. (**D**) A549 cells were treated with various concentrations of GMI (0, 4, 16 μg/mL) in the presence or absence of TNF-α for 9 h. Cells were pretreated with GMI for 1 h followed by the addition of 10 ng/mL of TNF-α. The isolated RNA samples were analyzed by semiquantitative RT-PCR, using the primers specific for MMP-9 and β-actin.

A549 cells (**Figure 1B**). We confirmed the same results in cells cotreated with GMI and 100 ng/mL TNF-α, demonstrating that GMI significantly blocks the invasiveness of TNF-α-induced A549 cells.

GMI Inhibits the Production of MMP-9 in TNF-α-Stimulated A549 Cells. TNF-α has been shown to induce MMP-9 in A549 cells in a previous study (*20*), as well as in our data (**Figure 2A**). The activities of MMP-9 were determined by gelatin zymography analysis. In **Figure 2A**, MMP-9 activities were mostly undetected in TNF-α-untreated medium. MMP-9 expression increased in a concentration-dependent manner in TNF-α-induced A549 cells. Therefore, we examined the effects of GMI on TNF-α-stimulated MMP-9 expression in A549 cells. The inhibitory activity of GMI on MMP-9 from supernatants, electrophoresed in gelatin SDS-PAGE, was evaluated. The MMP-9 containing gel slices were incubated at various concentrations of GMI (4, 16 μg/mL) in the serum-free medium 1 h before treatment with indicated dosages of TNF-α. After 24 or 48 h of incubation, the cells were harvested and the conditional media were subjected to gelatin zymography. The zymography assay revealed that TNF-α stimulation causes a substantial increase in the production of MMP-9 and that GMI treatment results in decreased levels of MMP-9 (**Figure 2B**). As shown in **Figure 2B**, the gelatinolytic activity of MMP-9 by TNF-α stimulation was inhibited by GMI in a dose-dependent manner.

GMI Reduces the Level of MMP-9 mRNA in TNF-α-Stimulated A549 Cells. To confirm our findings that GMI inhibits TNF-α-stimulated MMP-9 expression in A549 cells as measured by gelatin zymography (**Figure 2B**), we investigated the effect of GMI on the level of MMP-9 mRNA. To reveal the effects of GMI on the transcriptional level of MMP-9 mRNA, we performed RT-PCR analysis. In **Figure 2C**, A549 cells were treated with various concentrations of TNF-α, and MMP-9 mRNA increased

in a dose-dependent manner. The levels of MMP-9 mRNA decreased according to dosage with GMI treatment only. Similar results were found in cells cotreated with GMI and TNF-α (**Figure 2D**). Under TNF-α-stimulated experimental conditions, the expression levels of MMP-9 decreased significantly with various doses of GMI.

Effect of GMI on the Transcriptional Activity of MMP-9 Promoter in TNF-α-Stimulated A549 Cells. To evaluate the effects of GMI on the MMP-9 promoter, we performed a transient transfection with the pGL3-MMP-9 promoter and analyzed the luciferase activities. A plasmid constructed with MMP-9 promoter (pGL3-MMP-9 promoter) was used in this study (*18*). The plasmid contained a luciferase reporter gene driven by a 710 bp segment from the 5′-promoter region of the MMP-9 gene to examine TNF-α-mediated MMP-9 promoter activation in A549 cells. As shown in **Figure 3**, the luciferase activity of the transfectants treated with GMI (4, 16 μg/mL) was reduced. On the other hand, the luciferase activity of TNF-α-stimulated transfected cells increased to 1.6 times that of unstimulated transfected cells.

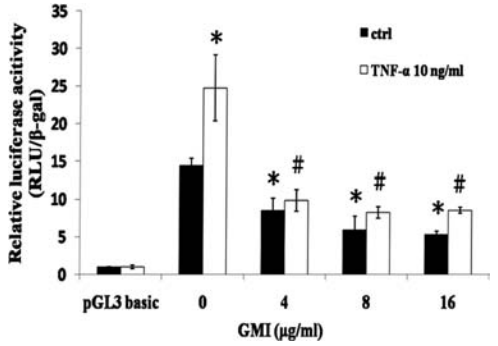

Figure 3. GMI inhibits TNF-α-induced promoter activity of MMP-9. Luciferase activity was measured in transiently transfected A549 cells using pWT-MMP-9 as described under Materials and Methods. The transfected cells were treated with GMI (0, 4, 8, 16 μg/mL) in the presence or absence of TNF-α (10 ng/mL). Cells were pretreated with GMI for 1 h followed by the addition of 10 ng/mL of TNF-α. The data are presented as mean ± SD of triplicate experiments. ∗, $p < 0.05$ vesus untreated control; #, $p < 0.05$ versus TNF-α-treated (10 ng/mL).

Moreover, in the presence of TNF-α stimulation, the luciferase activities of MMP-9 promoter were reduced to levels similar to those of unstimulated transfectants by various doses of GMI. A significant decrease ($p < 0.05$) in the reporter activity was observed. These results confirmed the inhibition by GMI on the gene expression of MMP-9 in TNF-α-stimulated A549 cells.

GMI Inhibits TNF-α-Induced MMP-9 Expression by Blocking NF-κB Activation. NF-κB has been shown to regulate the expression of several genes, the products of which are involved in lung inflammation and tumorigenesis, including MMP-9 (*15*). NF-κB constitutive activation is related to progression of various malignant neoplasms by carcinogens, inflammatory agents, and tumor promoters, such as cigarette smoke, H_2O_2, and TNF-α (*21*). In **Figure 3**, MMP-9 promoter activities are reduced by GMI treatment with TNF-α stimulation. We investigated which of the transcription factors are involved in the inhibition of the MMP-9 transcription by GMI in A549 cells. The MMP-9 promoter fragment between the −710 and −11 bp region contains several consensus motifs for NF-κB, AP-1, and Sp-1 transcription factors (*22*). NF-κB is a ubiquitous transcription factor that controls the expression of genes involved in immune responses, apoptosis, and cell cycle. Incorrect regulation of NF-κB may cause inflammatory and autoimmune diseases, viral infection, and cancer. As NF-κB has been shown to be activated by most carcinogens, inflammatory agents, and tumor promoters, including TNF-α, we speculated that GMI modulates MMP-9 promoter by mediation of NF-κB. We examined the inhibitory effect of GMI on the binding of NF-κB isolated from TNF-α-induced A549 cells to oligonucleotides that contain the sequence for the NF-κB binding sites from the MMP-9 promoter using EMSA (**Figure 4A**). As GMI suppressed TNF-α-induced NF-κB binding to MMP-9 promoter on in vitro EMSA, we investigated whether GMI-suppressed NF-κB complexes interact in vivo binding assay with the MMP9 promoter. We performed ChIP assays on A549 cells that were either untreated or treated with GMI and TNF-α induced. GMI treatment decreased NF-κB p65 binding to the NF-κB site of MMP-9 promoter in vivo binding assay with or without TNF-α treatment (**Figure 4B**). Together, these results demonstrate that GMI suppresses TNF-α-mediated MMP-9 transcription through NF-κB control.

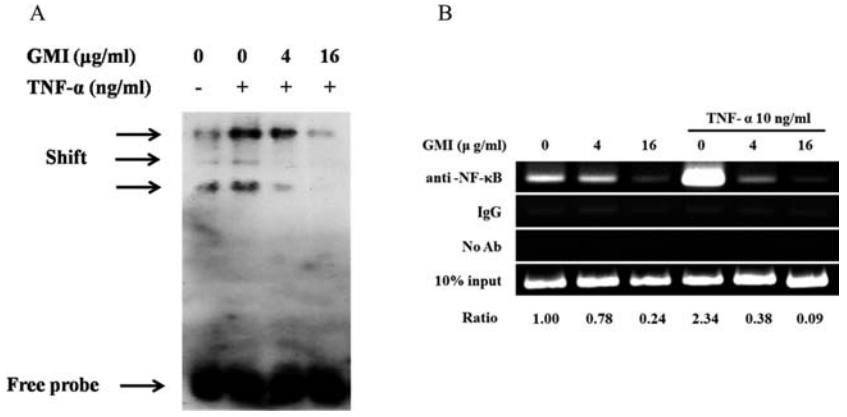

Figure 4. Effect of GMI on NF-κB binding activities in TNF-α-mediated stimulation of MMP-9 expression by ChIP assay or EMSA. (**A**) A549 cells were incubated with the indicated concentrations of GMI for 1 h and treated with TNF-α 10 ng/mL for 8 h. After incubation, nuclear extracts from the cells were analyzed by EMSA for the activated NF-κB using biotin-labeled oligonucleotide probes, respectively. (**B**) After serum starvation, A549 cells were incubated with the indicated concentrations of GMI for 1 h and treated with TNF-α 10 ng/mL for 8 h, and then cross-linked chromatin was prepared and immunoprecipitated with or without antibody (Ab) and IgG to NF-κB p65 before amplification of the MMP-9 gene region containing the NF-κB site. Immunoprecipitates were analyzed by PCR for the presence of the MMP-9 gene promoter sequence using the primer pair described under Materials and Methods. The DNA purified from the sonicated chromatin was directly analyzed by PCR using the same primer set, which served as an input control (input). Ratio means [anti-NF-κB/10% input of GMI 0 μg/mL]/[anti-NF-κB/10% input of TNF-α 10 ng/mL].

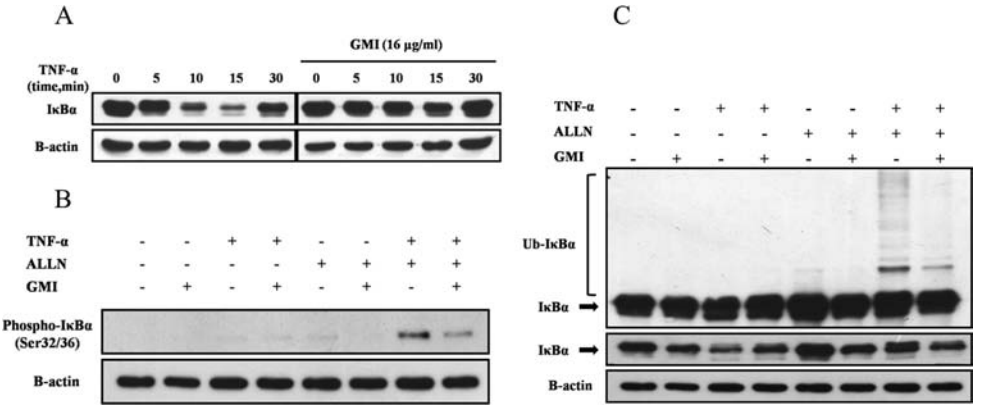

Figure 5. (**A**) Effect of GMI on TNF-α-induced degradation of IκBα. Cells (2×10^6 cells/100 mm) were incubated with 16 μg/mL GMI for 8 h and treated with 10 ng/mL TNF at 37 °C for the indicated times. Cytoplasmic extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to PVDF membranes. Western blot analysis was performed using anti-IκBα antibody. The loading control was blotted with anti-β -actin antibody. (**B**) Effect of GMI on the phosphorylation of IκBα by TNF-α. Cells (2×10^6 cells/100 mm) were preincubated with 16 μg/mL GMI for 8 h, incubated with 50 μg/mL *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then treated with 10 ng/mL TNF-α for 15 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phosphospecific IκBα (Ser 32/36) antibody. (**C**) Effect of GMI on TNF-α-induced ubiquitination of IκBα. Cells (2×10^6 cells/100 mm) were pretreated with 16 μg/mL GMI for1 h and then treated with 50 μg/mL ALLN for 30 min before exposure to 10 ng/mL TNF-α for 15 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using anti-IκBα antibody (for short- and long-time exposure).

GMI Inhibits TNF-Dependent Phosphorylation and Degradation of IκBα. In addition, NF-κB is a transcription factor and functions as a regulator of κB light chain expression in mature B lymphocytes and plasma cells . NF-κB is widely present as a heterotrimer of p50, p65, and IκB in the cytoplasm of all cells, where it is kept in an inactive state by a family of ankyrin domain-containing proteins, which include IκBβ, IκBγ, IκBε, Bcl-3, p105, and p100. Only when translocated to the nucleus is the sequence of events leading to activation initiated. The activation of NF-κB involves the phosphorylation, ubiquitination, and degradation of IκBα and phosphorylation of p65, which in turn leads to the translocation of NF-κB to the nucleus, where it binds to specific response elements in the DNA. In the NF-κB control pathway, IκBα is a major inhibitory subunit associated with the NF-κB complex. NF-κB translocation to the nucleus is accompanied by phosphorylation, ubiquitination, and degradation of IκBα. In our study, to determine whether GMI inhibits TNF-α-induced NF-κB activation in association with IκBα degradation, we pretreated A549 cells with GMI and then exposed them to TNF-α at various time points. We detected TNF-α-induced IκBα degradation in untreated GMI cells as early as 10−15 min, and resynthesis occurred 60 min after TNF-α treatment (**Figure 5A**). These results indicated that GMI mediates its effect via suppression of TNF-α-induced IκBα degradation, which eventually leads to suppression of NF-κB activation.

To understand whether the suppression of TNF-α-induced IκBα degradation is caused by inhibition of IκBα phosphorylation and ubiquitination, we used the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) to block degradation of IκBα. From analysis of cytoplasmic extract and recognition of the serine-phosphorylated form of IκBα antibody, TNF-α-induced IκBα phosphorylation (**Figure 6B**) and TNF-α-induced IκBα ubiquitination (**Figure 6C**) were suppressed by GMI. The results showed that GMI inhibits TNF-α-induced IκBα degradation through phosphorylation and ubiquitination pathways.

GMI Inhibits TNF-α-Induced Phosphorylation and Nuclear Translocation of NF-κB p65. We also investigated the effect of GMI on the TNF-α-induced phosphorylation of NF-κB p65, because phosphorylation is required for the transcriptional activity of NF-κB p65. In general, NF-κB is located in cytoplasm in the untreated state. TNF-α induced phosphorylation of NF-κB p65 in a time-dependent manner, and GMI suppressed it (**Figure 6A**). On immunocytochemistry analysis, GMI suppressed TNF-α-induced nuclear translocation of NF-κB p65 (**Figure 6B**).

NF-κB has been shown to regulate the expression of several genes, the products of which are involved in lung inflammation and tumorigenesis, including MMP-9. NF-κB constitutive activation is related to the progression of various malignant neoplasms by carcinogens, inflammatory agents, and tumor promoters, such as cigarette smoke, H_2O_2, and TNF-α (*21*). Reduction of NF-κB activation by GMI is due to inhibition of IκBα phosphorylation and degradation, as well as suppression of p65 phosphorylation and translocation to the nucleus. These results imply the importance of GMI in suppression of invasive activity through the NF-κB/MMP-9 pathway.

MMP-9/gelatinase B is a putative therapeutic target of COPD and multiple sclerosis (*23*). MMP-9 is a case in point: its dramatic overexpression in cancer and various inflammatory conditions clearly points to the molecular mechanisms controlling its expression as a potential target for eventual rational therapeutic intervention (*24*). There are many transcriptional factors that can modulate the MMP-9 promoter including nuclear factor (NF)-κB, activator protein (AP)-1, and Sp-1 transcription factors. In our study, the MMP-9 promoter is consistently and highly activated without TNF-α activation; it seems that GMI also can regulate other factors (such as AP-1 or Sp-1) to affect the MMP-9 promoter activity. We found that GMI suppresses the expression of MMP-9. We also showed that TNF-α-induced invasion of tumor cells is suppressed by GMI. Overall, these results suggest that GMI has potential in the prevention and treatment of lung inflammation or cancer. In our study, GMI was generated from *G. microsporum* and homologues to FIP-*gts*, which is one class of *G. lucidum*. From previous papers, *G. lucidum* inhibits expression of urokinase-type plasminogen activator (uPA) and its receptor uPAR through constitutively active transcription factors NF-κB and AP-1. *G. lucidum* also suppresses cell adhesion and cell migration of highly invasive breast and prostate cancer cells (*25*). Another study demonstrated that *G. lucidum* inhibits the growth of MDA-MB-231 breast cancer cells through inhibition of Akt/NF-κB signaling (*26*). In recent years, it has been shown that prefeeding with *G. lucidum* antlered form (AF)-containing diet suppresses lung metastasis in cyclophosphamide

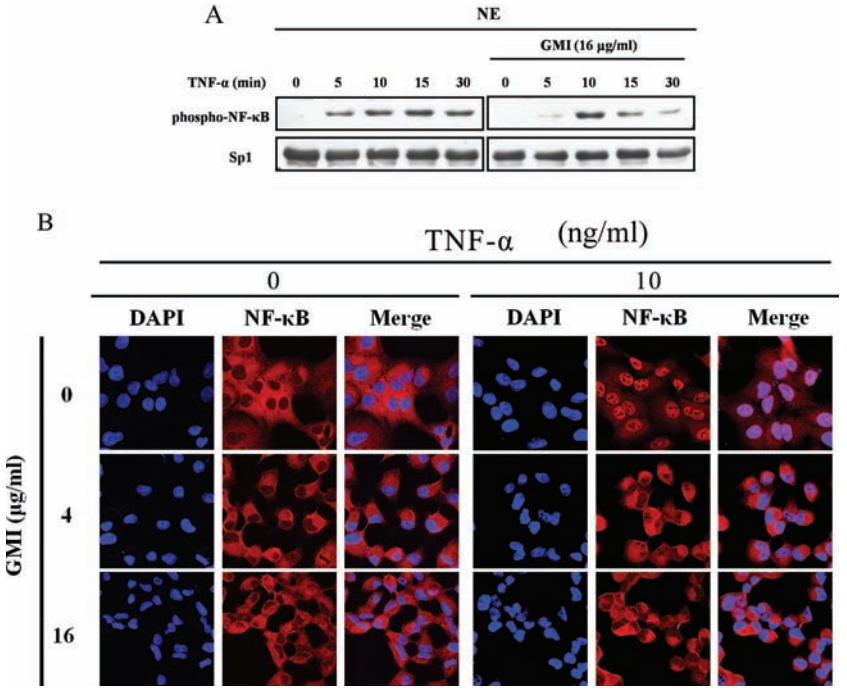

Figure 6. Effects of GMI on phosphorylation and nuclear translocation of NF-κB p65 induced by TNF-α. (**A**) GMI inhibits TNF-α-induced translocation and phosphorylation of NF-κB p65. A549 cells were either untreated or pretreated with 16 μg/mL GMI for 8 h at 37 °C and then treated with 10 ng/mL TNF-α for the indicated times. Cytoplasmic and nuclear extracts were prepared and analyzed by Western blotting using phosphospecific NF-κB p65 antibodies. For loading control of nuclear protein, the membrane was reblotted with either β-actin or anti-Sp1 antibody. GMI inhibits TNF-α-induced nuclear translocation of p65. (**B**) Representative immunostaining using confocal microscopy is shown for A549 cell treatment with various dosages of GMI for 8 h and then exposed to 10 ng/mL TNF-α for 20 min. After treatment, immunocytochemical analysis was performed as described under Materials and Methods.

(CY)-induced C57BL/6 mice injected with Lewis lung carcinoma (LLC). In particular, the number of nodules in the *G. lucidum* AF-fed group injected with LLC 7 days after CY administration was significantly lower compared to that of the control group receiving no *G. lucidum* (*27*). From these results, it is clear that *G. lucidum* possesses antimetastatic and anti-invasive activities. We also demonstrated that GMI inhibits TNF-α-induced invasion by modified Boyden chamber via down-regulation of NF-κB/MMP-9 activity.

NF-κB activation has been shown to cause the expression of several gene products involved in the processes of cell survival, proliferation, apoptosis, invasion, metastasis, and angiogenesis. MMP-9 is one of the genes controlled by NF-κB. In recent papers, astilbin inhibition of T lymphocyte adhesion is related to the reduction of CD44 expression and TNF-α production, which may cause decreased MMP-9 secretion (*28*). Curcumin also has anti-inflammatory properties and may prevent the migration of human aortic smooth muscle cells (HASMCs) by suppressing MMP-9 expression through down-regulation of NF-κB (*29*). Tanshinone IIA inhibits the migration and MMP-9 activity of TNF-α-induced HASMCs through IκBα phosphorylation and p65 nuclear translocation (*30*). In our study, we showed similar results of TNF-α-induced MMP-9 activity suppressed by GMI through inhibition of the translocation of NF-κB.

Our results clearly demonstrate that anti-invasive and anti-inflammatory activities of GMI with or without TNF-α stimulation in A549 cells are mediated through the inhibition of the IκBα/NF-κB/MMP-9 pathway. These findings suggest that GMI has considerable potential for the treatment of lung inflammation and COPD, as well as in cancer chemoprevention.

ABBREVIATIONS USED

FIP, fungal immunomodulatory protein; GMI, immunomodulatory protein from *Ganoderma microsporum*; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility-shift assay; ChIP, chromatin immunoprecipitation assay; MMP-9, matrix metalloproteinase-9; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal.

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