Mori folium inhibits interleukin-1β-induced expression of matrix metalloproteinases and inflammatory mediators by suppressing the activation of NF-κB and p38 MAPK in SW1353 human chondrocytes

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Abstract. The pro-inflammatory cytokine interleukin-1β (IL-1β) is known to play a crucial role in the pathogenesis of osteoarthritis (OA) by stimulating several mediators that contribute to cartilage degradation. Mori folium, the leaves of *Morus alba* L., has long been used in traditional medicine to treat diabetes, protect the liver, and lower blood pressure; however, the role of Mori folium in OA is not yet fully understood. Therefore, in the present study, we investigated whether Mori folium water extract (MF) inhibited the catabolic effects of IL-1β *in vitro*, and also whether it inhibited the matrix metalloproteinases (MMPs), inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) through the attenuation of nuclear factor-κB (NF-κB) and mitogen activated protein kinase (MAPK) pathways in SW1353 human chondrocytes. MMP proteins in culture medium were determined using a cytokine-specific enzyme-linked immunosorbent assay (ELISA). The production of NO and prostaglandin E_2 (PGE_2) were evaluated using Griess reagent and ELISA. Subsequently, the mRNA and protein levels of MMPs, iNOS, COX-2, NF-κB and MAPKs were examined by RT-qPCR and/or western blot analysis. The results indicate that MF significantly reduced the IL-1β-induced release of MMP-1 and -13 in SW1353 cells, which was associated with the inhibition of MMP-1 and -13 mRNA and protein expression in a concentration-dependent manner at concentrations with no cytotoxicity. MF also attenuated the IL-1β-induced production of NO and PGE_2, and reduced iNOS and COX-2 expression. Furthermore, we noted that MF markedly suppressed the IL-1β-induced nuclear translocation of NF-κB, which correlated with the inhibitory effects of MF on inhibitor-κB (IκB) degradation, and the phosphorylation of p38 MAPK was selectively restored by MF upon IL-1β stimulation. These results indicate that MF inhibited the production and expression of MMP-1 and -13 and inflammatory mediators, at least in part, through suppressing the activation of either NF-κB or p38 MAPK in IL-1β-treated SW1353 chondrocytes. Therefore, the novel findings of the present study suggest that MF is a potential therapeutic choice for chondroprotection against the collagen matrix breakdown in the cartilage of diseased tissues, such as those found in patients with arthritic disorders.

Introduction

Currently, osteoarthritis (OA) is a significant clinical issue worldwide, and this situation is expected to worsen with the growth of aging populations (1,2). OA, also known as degenerative arthritis, degenerative joint disease, or osteoarthrosis, is a type of chronic and degenerative joint disease characterized by an increase in protease activity, which results in the degradation of critical extracellular matrix (ECM) proteins as well as pain which results in disabilities (1,2).

In addition to mechanical and genetic factors, chronic and excessive production of inflammatory cytokines and mediators is an important characteristic of OA (2,3). Of the inflamma-

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tory cytokines, interleukin-1β (IL-1β) is considered to be one of the most potent catabolic factors in arthritis (3-5). IL-1β plays a pivotal role in the destruction of the cartilage matrix by upregulating the production of proteases, such as matrix metalloproteinases (MMPs) and zinc-dependent endopeptidases, which are specifically controlled by the tissue inhibitors of matrix metalloproteinases (TIMPs) (3,6). In healthy cartilage, there is homeostasis between MMPs and TIMPs, which is disturbed in OA (2,6). In the joint cartilage, MMPs are synthesized and secreted by the residing chondrocytes, particularly under arthritic conditions (6,7). Of the various MMPs, collagenases such as MMP-1, -8 and -13, which are also known as collagenase-1, -2 and -3, are of particular importance, as they are elevated in joint disorders and closely related to pathological conditions, such as joint inflammation and joint degenerative diseases (6). Thus, reducing the activity of collagenases may have beneficial results, in the form of chondroprotection against pathological conditions such as OA.

It is also well documented that the expression of collagenases in chondrocytes is regulated by the activation of mitogen-activated protein kinase (MAPK) family members, including c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK), and several transcription factors, including nuclear factor-κB (NF-κB), which is known to be activated in human chondrocytes (8-10). In addition, IL-1β induces excessive inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in chondrocytes, leading to the elevated production of NO and prostaglandin E_2 (PGE_2), which accelerate cartilage degradation by inhibiting proteoglycan biosynthesis (11,12). Accordingly, NO and PGE_2, which are found in high levels in the synovial fluid of OA patients, are also regarded as beneficial therapeutic targets in the treatment of OA; thus, non-steroidal anti-inflammatory drugs (NSAIDs) have been used for treatment for several years. However, adverse effects, particularly gastrointestinal diseases and cardiovascular risks, are commonly associated with these agents (13,14). Therefore, there is currently considerable interest in the development of new drugs from natural sources, which can be safely used for the prolonged treatment of OA.

For thousands of years, herbal medicines have been used safely and effectively for alleviating and treating various diseases in many countries; there is increasing interest in the pharmacological activity of traditional herbs that are widely used in traditional medicine, and numerous studies support their potential clinical benefits for diseases that are difficult to treat (15,16). Among them, *Morus alba* L., or white mulberry, a deciduous tree that belongs to the Moraceae family, is widely distributed in Asia (16). In particular, Mori folium, the leaves of *Morus alba* L., is one of the most widely consumed medicinal herbs in Asia, including Korea, and medications made from Mori folium have been found to be useful in the treatment of metabolic disorders, such as diabetes, hyperlipidemia, and high blood pressure (17-19). Previous research has shown that Mori folium exerts diverse pharmacological effects, including anti-microbial (20), anti-inflammatory (21), antioxidant (22), antitumor (23,24), anti-atherosclerotic (25), anti-obesity (17,26), anti-hypotensive (25), and anti-hypoglycemic effects (21), and has the potential to protect the liver (25,27). To the best of our knowledge, no study has been conducted to date on the potential chondroprotective effects and cellular action mechanisms of Mori folium. In this study, as a part of our research on novel biologically active substances for the prevention and treatment of OA from traditional medicinal resources, we investigated the potential of Mori folium water extract (MF) on the production of MMPs and inflammatory mediators in IL-1β-stimulated SW1353 human chondrocyte cells.

Materials and methods

Preparation of MF. The leaves of *Morus alba* L., Mori folium, were obtained from Bio-Port Korea, Inc. (Busan, Korea) and authenticated by Professor Su Hyun Hong, Department of Biochemistry, Dongeui University College of Korean Medicine (Busan, Korea). The dried leaves were subsequently cut into small pieces, ground into a fine powder, and then boiled with distilled water (50 g/500 ml) for 3 h. The extract was filtered through Whatman no. 3 filter paper (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) twice in order to remove any insoluble materials, and the filtrate was lyophilized and then crushed into a thin powder. The extracts (MF) were dissolved in a 100 mg/ml concentration with distilled water, and the stock solution was then diluted with Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) to the desired concentration prior to use.

Cell culture. SW1353 chondrocytes were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) which contained 10% v/v fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in the presence or absence of MF at 37˚C in humidified air with 5% CO_2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was measured using an MTT assay. Briefly, SW1353 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well. After incubation for 24 h, the cells were treated with various concentrations of the MF or 40 ng/ml IL-1β (R&D Systems, Inc., Minneapolis, MN, USA) individually or were pretreated with different concentrations of MF for 1 h before the IL-1β treatment. After 24 h, the medium was removed, and the MTT working solution (0.5 mg/ml; Sigma-Aldrich Chemical Co.) was then added to the culture plates and incubated continuously at 37˚C for 2 h. Subsequently, the culture supernatant was removed from the wells, and dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemical Co.) was added to dissolve the formazan crystals. After shaking, the absorbance of each well was measured at 540 nm with a microplate reader (Dynatech Laboratories, Chantilly VA, USA), and the results were expressed as cell viability relative to the untreated control, which was considered 100% viable.

Enzyme-linked immunosorbent assay (ELISA). The inhibitory effects of MF on the production of MMPs (MMP-1, -2, -3 and -13) and PGE_2 were examined using commercial ELISA kits (purchased from R&D Systems, Inc.). Briefly, cells were treated with IL-1β (40 ng/ml) only or alternatively were pretreated with various concentrations of MF for 1 h before IL-1β treatment. After 24 h, the concentrations of the MMPs and PGE_2 in the culture medium were determined using selective ELISA kits, according to the manufacturer's instructions.

Table I. Sequences of the primer pairs employed in the RT-qPCR reactions.

Gene name	Sequence
MMP-1	
Sense	5'-CTG-TTC-AGG-GAC-AGA-ATG-TGC-3'
Antisense	5'-TTG-GAC-TCA-CAC-CAT-GTG-TT-3'
MMP-2	
Sense	5'-GTC-AGT-GAG-AAG-GAA-GTG-GAC-TCT-3'
Antisense	5'-ATG-TTC-TTC-TCT-GTG-ACC-CAG-TC-3'
MMP-3	
Sense	5'-TGC-GTG-GCA-GTT-TGC-TCA-GCC-3'
Antisense	5'-GAA-TGT-GAG-TGG-AGT-CAC-CTC-3'
MMP-13	
Sense	5'-GGC-TCC-GAG-AAA-TGC-AGT-CTT-TCT-T-3'
Antisense	5'-ATC-AAA-TGG-GTA-GAA-GTC-GCC-ATG-C-3'
iNOS	
Sense	5'-GTG-AGG-ATC-AAA-AAC-TGG-GG-3'
Antisense	5'-ACC-TGC-AGG-TTG-GAC-CAC-3'
COX-2	
Sense	5'-TCA-GCC-ACG-CAG-CAA-ATC-CT-3'
Antisense	5'-GTG-ATC-TGG-ATG-TCA-CG-3'
GAPDH	
Sense	5'-CGG AGT CAA CGG ATT TGG TCG TAT-3'
Antisense	5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'

MMP, matrix metalloproteinase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from the cultured cells was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using AccuPower® RT PreMix (Bioneer, Daejeon, Korea) containing moloney murine leukemia virus reverse transcriptase. RT-generated cDNA was amplified by PCR using selected primers (Table I), which were purchased from Bioneer. After amplification, the PCR reactions were electrophoresed in 1% agarose gels and visualized using ethidium bromide (EtBr; Sigma-Aldrich Chemical Co.) staining. In a parallel experiment, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Protein extraction and western blot analysis. For total protein extraction, the cells were harvested and lysed in lysis buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet-P40 (NP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM dithiothreitol (DTT)] for 1 h. In a parallel experiment, nuclear and cytosolic proteins were prepared using nuclear extraction reagents (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The insoluble materials were discarded by centrifugation at 13,000 x g for 20 min at 4˚C. Protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. For the western blot analysis, equivalent amounts of proteins were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). After blocking with 5% skimmed milk, the membranes were incubated with protein specific antibodies [MMP-1 (1:1,000; ab52631, rabbit monoclonal; Abcam, Cambridge, UK), MMP-2 (1:1,000; SC-10736, rabbit polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), MMP-3 (1:1,000; ab52915, rabbit monoclonal; Abcam), MMP-13 (1:1,000; ab51072, rabbit monoclonal; Abcam), iNOS (1:500; SC-7271, mouse monoclonal, Santa Cruz Biotechnology, Inc.), COX-2 (1:500; SC-19999, mouse monoclonal; Santa Cruz Biotechnology, Inc.), NF-κB p65 (1:500; SC-109, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), IκB-α (1:500; SC-371, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), ERK (1:1,000; SC-154, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), p-ERK (1:500; #9106S, mouse monoclonal; Cell Signaling Technology, Inc., Danvers, MA, USA), p38 (1:1,000; SC-535, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), p-p38 (1:500; #9211S, rabbit polyclonal; Cell Signaling Technology, Inc.), JNK (1:1,000; #9252S, rabbit polyclonal; Cell Signaling Technology, Inc.), p-JNK (1:500; #9255S, mouse monoclonal; Cell Signaling Technology, Inc.), Lamin B (1:500; SC-6216, goat polyclonal; Santa Cruz Biotechnology, Inc.) and β-actin (1:1,000; sc-1616, goat polyclonal; Santa Cruz Biotechnology, Inc.)] for 1 h, subsequently incubated with appropriate enzyme-linked secondary antibodies [mouse IgG, HRP-linked whole antibody (NA931) and rabbit IgG, HRP-linked whole antibody (NA934); Amersham Corp., Arlington Heights, IL, USA], and visualized using an enhanced chemiluminescence (ECL) solution (Amersham Corp.), according to the manufacturer's instructions. The primary antibodies were purchased from Santa Cruz Biotechnology, Inc. and Abcam.

Measurement of NO production. The concentration of NO in the culture supernatants was determined by measuring nitrite, a stable oxidation product of nitric oxide, using Griess reagent (Sigma-Aldrich Chemical Co.). The cell culture conditions were the same as those used for the ELISA. Following incubation with IL-1β and/or MF for 24 h, 100 μl of each culture supernatant was mixed with an equal volume of Griess reagent for 10 min at room temperature. Subsequently, absorbance was measured at 540 nm with a microplate reader, and nitrite production was determined by $NaNO_2$ standard curve, as previously described (28).

Immunofluorescence staining. For the detection of the translocation of NF-κB p65, the SW1353 cells were grown on glass coverslips in 6-well plates for 24 h. Cells were pretreated with MF (800 μg/ml) for 1 h prior to stimulation with IL-1β (40 ng/ml). Following incubation for 30 min, the cells were washed twice with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde, treated with 0.2% Triton X-100, and subsequently blocked with 2% bovine serum albumin. The cells were sequentially incubated with an anti-NF-κB p65 antibody (SC-109; Santa Cruz Biotechnology, Inc.) and fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (IgG; Cat. no. 711-001-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). After washing with PBS, the nuclei were stained with 4'6-diamidino-2-phenylindole

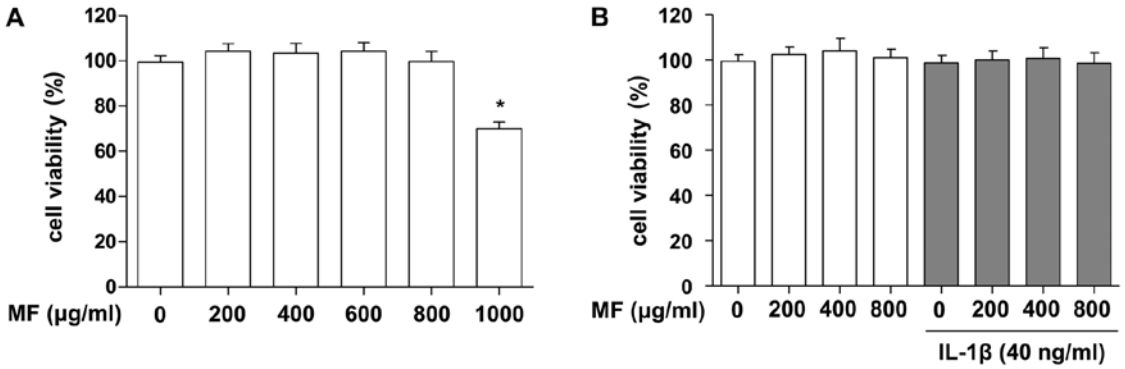

Figure 1. Effects of Mori folium water extract (MF) and interleukin-1β (IL-1β) on the cell viability of SW1353 chondrocytes. (A) Cells were treated with the indicated concentrations of MF alone or (B) pretreated with MF for 1 h before IL-1β (40 ng/ml) treatment. After 24 h, cell viability was assessed using an MTT reduction assay. Data are expressed as the means ± standard deviation of the three independent experiments (*p<0.05 vs. untreated control).

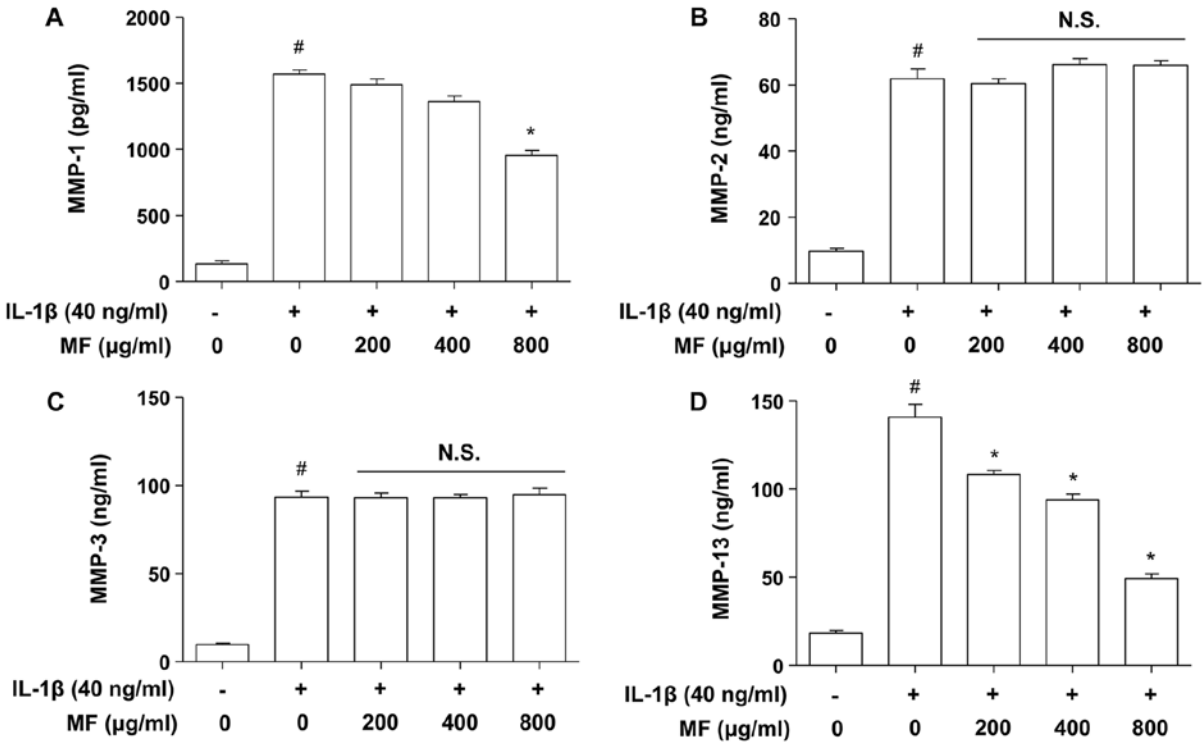

Figure 2. Effects of Mori folium water extract (MF) on the release of (A) matrix metalloproteinase (MMP)-1, (B) MMP-2, (C) MMP-3, and (D) MMP-13 on interleukin-1β (IL-1β)-stimulated SW1353 chondrocytes. Cells were pretreated with various concentrations of MF for 1 h before incubation with IL-1β (40 ng/ml) for 24 h. The concentrations of the MMPs were measured in culture media using commercial ELISA kits. The values represent the means ± SD of three independent experiments (#p<0.05 compared with control group; *p<0.05 compared with IL-1β-treated group; N.S., not significant).

(DAPI; Sigma-Aldrich Chemical Co.), and fluorescence was visualized using a fluorescence microscope (Carl Zeiss, Jena, Germany), as previously described (29).

Statistical analysis. The values are expressed as the means ± SD. A Student's t-test was used to evaluate the differences between the samples treated with IL-1β only and the samples treated with IL-1β and MF. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Effects of MF on the viability of SW1353 chondrocytes. To evaluate whether or not MF exerted cytotoxic effects on SW1353 cells, the cells were treated with various concentrations of MF for 24 h. In the MTT assays, we noted that MF concentrations up to 800 μg/ml did not induce cytotoxicity in the SW1353 cells, whereas 1,000 μg/ml MF significantly reduced viability (approximately 70%; Fig. 1A). In a subsequent experiment, when we administered MF to IL-1β-treated SW1353 cells at concentrations below 800 μg/ml, no adverse effects on cell viability were noted (Fig. 1B). Therefore, concentrations of MF up to 800 μg/ml were used in the remaining experiments.

Suppressive effects of MF on IL-1β-induced production of MMP-1 and -13 in SW1353 chondrocytes. Previous studies have suggested that various MMPs are involved in both physiological collagen turnover in articular cartilage and matrix

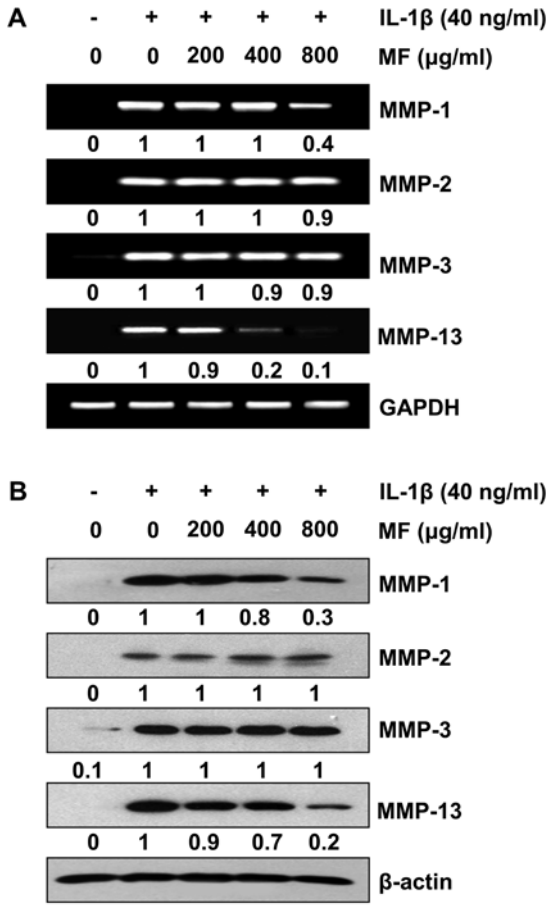

Figure 3. Effects of Mori folium water extract (MF) on the expression of matrix metalloproteinases (MMPs) in interleukin-1β (IL-1β)-stimulated SW1353 chondrocytes. (A) Cells were pretreated with different concentrations of MF for 1 h before incubation with IL-1β (40 ng/ml) for 24 h. The total RNAs were prepared for RT-qPCR analysis of the MMP mRNA expression using the indicated primers. (B) The cells were lysed after a 24-h treatment culture, and the cellular proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with the specific antibodies against MMPs. Proteins were visualized using the ECL detection system. GAPDH and β-actin were used as internal controls for the RT-qPCR and western blot analysis, respectively. The relative ratios of expression from RT-qPCR and western blot analysis are presented at the bottom of each of the results as relative values of the GAPDH and β-actin expression, respectively.

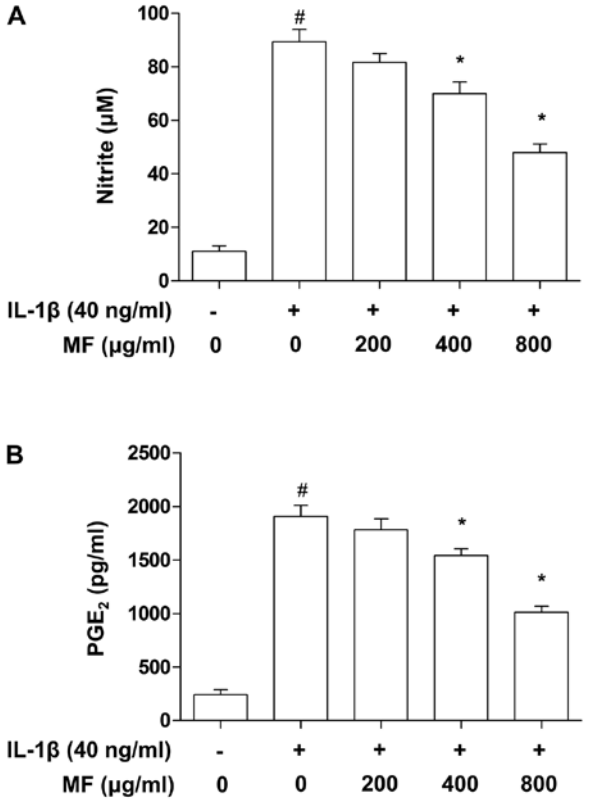

Figure 4. Inhibition of nitric oxide(NO) and prostaglandin E2 (PGE_2) production by Mori folium water extract (MF) in interleukin-1β (IL-1β)-stimulated SW1353 chondrocytes. The cells were pretreated with different concentrations of MF for 1 h before incubation with IL-1β (40 ng/ml). (A) Following treatment for 24 h, the supernatants were prepared, and the nitrite content was measured using Griess reagent. (B) The amount of PGE_2 was measured in the culture media using a commercial ELISA kit. Each value represents the means ± standard deviation of the cultures (triplicate) ($^{\#}p<0.05$ compared with the control group; $^{*}p<0.05$ compared with the IL-1β-treated group).

degradation in OA cartilage (2,6). Therefore, to examine the inhibitory effect of MF on IL-1β-induced MMP production, SW1353 cells were stimulated with IL-1β (40 ng/ml) for 24 h in the presence and absence of MF, and subsequently the release of MMPs from SW1353 cells was detected in the culture supernatants using ELISA. As also reported previously (30,31), increased release of MMPs (MMP-1, -2, -3 and -13) was detected in the culture supernatants after stimulation with IL-1β (Fig. 2); however, pretreatment of the SW1353 cells with MF dose-dependently reduced the production of IL-1β-stimulated collagenases MMP-1 and -13, but not MMP-2 (gelatinase A) or -3 (a stromelysin), and levels were comparable to those found in cells not treated with MF.

Inhibition of IL-1β-induced expression of MMP-1 and -13 by MF in SW1353 chondrocytes. To evaluate the effects of MF on IL-1β-induced expression of MMPs in SW1353 cells, cells were treated with IL-1β alone or with different concentrations of MF for 24 h. RT-qPCR and western blot analysis revealed that the levels of all MMPs in the SW1353 cells treated with IL-1β alone markedly increased compared to the levels detected in the untreated cells (Fig. 3); however, pretreatment with MF was found to inhibit the increase in MMP-1 and -13 but not MMP-2 and -3 caused by treatment with IL-1β in a concentration-dependent manner. These results suggest that MF is a potent inhibitor of IL-1β-mediated MMP-1 and -13 transcription in SW1353 cells, whereas it appears not to interfere with MMP-2 and -3 gene expression.

Attenuation of IL-1β-induced NO and PGE_2 production by MF in SW1353 chondrocytes. It is well-known that inflammatory mediators, such as NO and PGE_2, play key roles in the progression of cartilage destruction in OA (32,33), and thus the effects of MF on the level of NO and PGE_2 in IL-1β-stimulated SW1353 cells were examined in this study. To verify the effects of MF on the levels of NO and PGE_2 produced by SW1353 cells, supernatants of treated cell cultures were collected and subjected to Griess reagent and ELISA, respectively. Consistent with previous reports (34,35), treating SW1353 cells with IL-1β alone markedly elevated the levels of NO and PGE_2 compared to the control group (Fig. 4); however, MF considerably abrogated the release of NO and PGE_2 in IL-1β-treated SW1353 cells in a concentration-dependent manner at a range of 200-800 μg/ml.

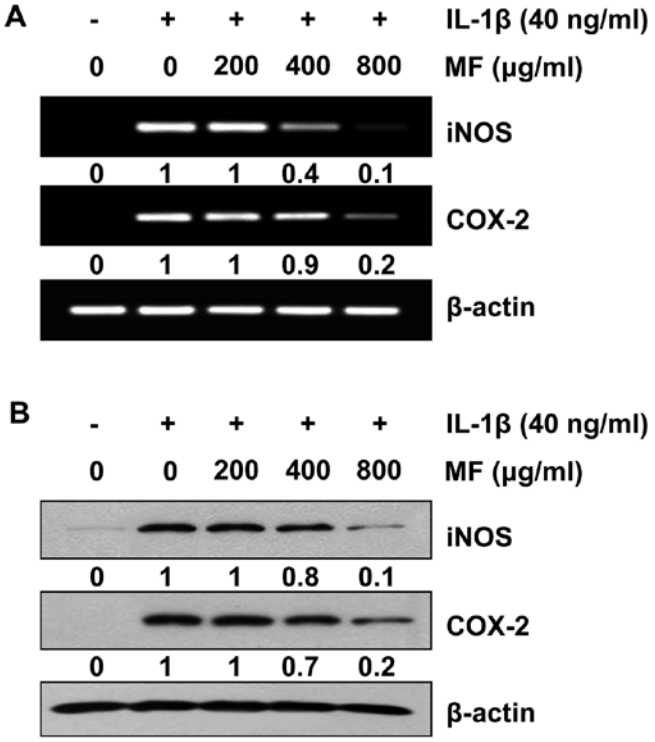

Figure 5. Inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by Mori folium water extract (MF) in interleukin-1β (IL-1β)-treated SW1353 chondrocytes. (A) The cells were pretreated with different concentrations of MF for 1 h before incubation with IL-1β (40 ng/ml). Following 24 h of treatment, total RNA was isolated, and RT-qPCR was performed using iNOS and COX-2 primers. (B) The cells were lysed after a 24-h treatment culture, and cellular proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then probed with specific antibodies against iNOS and COX-2. The proteins were subsequently visualized using an ECL detection system. GAPDH and β-actin were used as the internal controls for the RT-qPCR and western blot analyses, respectively. The relative ratios of expression in the results of the RT-qPCR and western blot analysis are presented at the bottom of the lanes as relative values of GAPDH and β-actin expression, respectively.

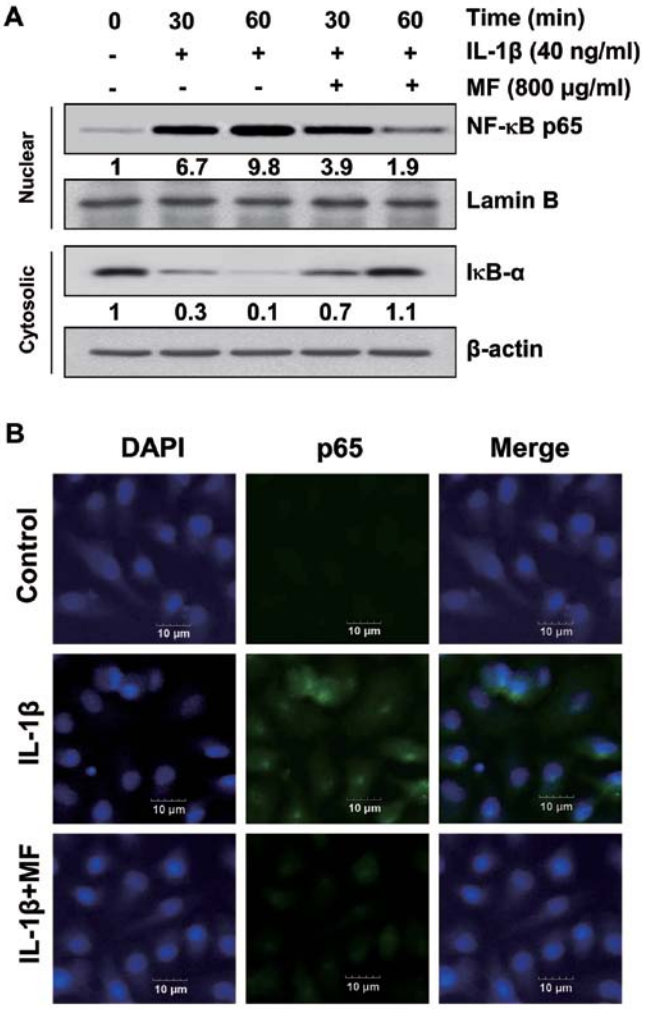

Figure 6. Inhibition of nuclear factor-κB (NF-κB) nuclear translocation by Mori folium water extract (MF) in interleukin-1β (IL-1β)-stimulated SW1353 chondrocytes. (A) The cells were pretreated with MF (800 μg/ml) for 1 h before the IL-1β treatment (40 ng/ml) for the indicated times. The nuclear and cytosolic proteins were run on 10% SDS-PAGE followed by western blot analysis using anti-NF-κB p65 and anti-IκB-α antibodies and an ECL detection system. Lamin B and β-actin were used as internal controls for the nuclear and cytosolic fractions, respectively. The relative ratios of expression in the results of the western blot analysis are presented at the bottom of the lanes as relative values of lamin B and β-actin expression, respectively. (B) The cells were pretreated with MF (800 μg/ml) for 1 h before IL-1β treatment (40 ng/ml). After 1 h of incubation, the localization of NF-κB p65 was visualized with fluorescence microscopy after immunofluorescence staining with anti-NF-κB p65 antibody (green). The cells were also stained with DAPI to visualize the nuclei (blue). The results are representative of those obtained from three independent experiments.

Reduction of IL-1β-induced iNOS and COX-2 expression by MF in SW1353 chondrocytes. Subsequently, RT-qPCR and western blot analysis were performed to determine whether or not the inhibition of NO and PGE_2 production by MF in the IL-1β-stimulated SW1353 cells was associated with decreased levels of iNOS and COX-2 expression. The results indicate that the IL-1β-induced increase in the iNOS and COX-2 mRNA levels was reversed by MF in a concentration-dependent manner (Fig. 5A). In a parallel experiment, the elevated protein levels of iNOS and COX-2 resulting from stimulation with IL-1β were also decreased following pretreatment with MF (Fig. 5B). These results indicate that the reduced expression of iNOS and COX-2 at the transcriptional levels contributed to the inhibitory effect of MF on IL-1β-induced NO and PGE_2 production.

Effect of MF on IL-1β-induced nuclear translocation of NF-κB in SW1353 chondrocytes. Previous studies have reported that IL-1β-induced NF-κB activation is involved in upregulating MMPs, iNOS and COX-2 transcriptional activities (36,37). Therefore, we determined whether or not the inhibitory effect of MF on the IL-1β-induced activation of MMPs, iNOS and COX-2 was mediated by the suppression of NF-κB signaling by measuring the nuclear translocation of NF-κB. Western blot analyses revealed that treatment with IL-1β enhanced the nuclear accumulation of NF-κB proteins within 30 min, concomitantly with the degradation of IκB-α in the cytosol (Fig. 6A); however, pretreatment with MF reduced the IL-1β-induced nuclear accumulation of NF-κB and the degradation of IκB-α compared to those in cells treated with IL-1β alone. The immunofluorescence images also revealed that the nuclear translocation of NF-κB p65 was strongly induced after the stimulation of cells with IL-1β, and the shift in NF-κB p65 to the nucleus was completely abolished after pretreating the cells with MF (Fig. 6B). These findings indicate that MF inhibits IL-1β-induced NF-κB activation in

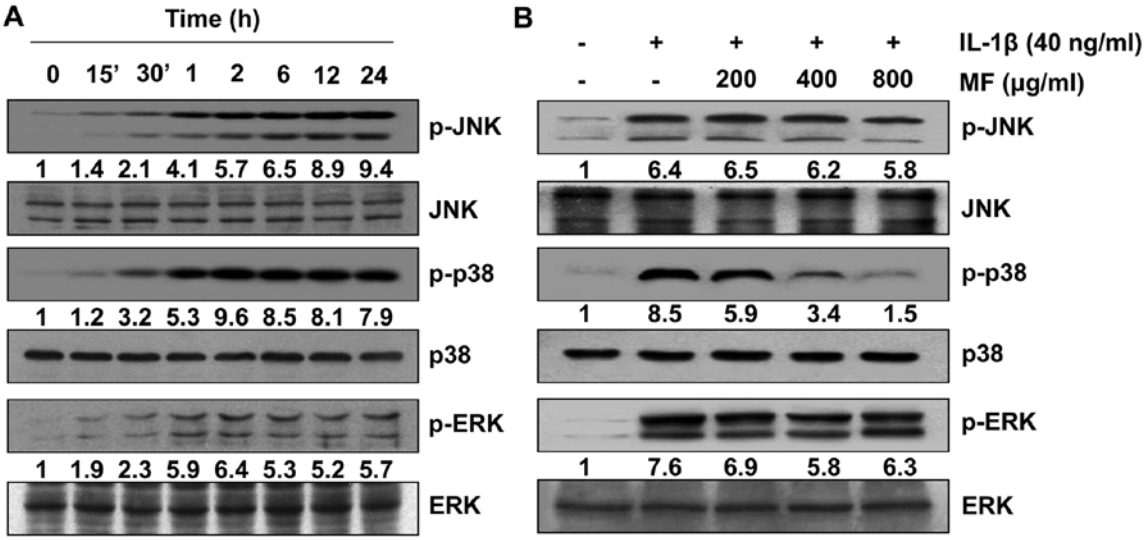

Figure 7. Effect of Mori folium water extract (MF) on interleukin-1β (IL-1β)-induced phosphorylation of mitogen activated protein kinases (MAPKs) in SW1353 chondrocytes. The cells were treated with IL-1β (40 ng/ml) alone (A) for the indicated times or (B) pretreated with various concentrations of MF for 1 h before IL-1β treatment (40 ng/ml) for 1 h. The proteins were isolated and subjected to SDS-PAGE followed by western blot analysis using the indicated antibodies and an enhanced chemiluminescence (ECL) detection system. The numbers represent the average densitometric analyses of p-MAPKs as compared with total MAPK expression.

SW1353 cells through the suppression of IκB degradation and the nuclear translocation of NF-κB.

Inhibition of IL-1β-induced activation of p38 MAPK by MF in SW1353 chondrocytes. As it is well-known that the MAPK pathway is involved in IL-1β-induced MMP and inflammatory mediator expression (10,38), we subsequently investigated whether or not the inhibitory effect of MF on their expressions was mediated by MAPK signaling cascades. Western blot analysis confirmed that IL-1β treatment enhanced the phosphorylation of JNK, p38 MAPK and ERK without markedly affecting their total protein levels (Fig. 7A). Therefore, the effect of MF on IL-1β-induced phosphorylation of MAPKs in SW1353 cells was evaluated. The results reveal that pretreatment with MF significantly reduced the IL-1β- stimulated activation of p38 MAPK to near the control levels in a concentration-dependent manner, but it had no such effect on the activity of JNK and ERK (Fig. 7B). The results suggest that the suppression of IL-1β-induced p38 MAPK activation by MF is responsible for the observed condroprotective effect of MF on IL-1β-stimulated SW1353 cells.

Discussion

In this study, we investigated whether or not MF inhibited the release and expression of MMPs and inflammatory mediators induced by a representative pro-inflammatory cytokine, IL-1β, in SW1353 chondrocytes. As the data demonstrated, pretreating SW1353 cells with MF effectively suppressed the IL-1β-mediated release of MMP-1, -3, NO and PGE_2, as indicated by the attenuation of their corresponding gene expressions, at least in part via blocking NF-κB and p38 MAPK activation.

It is well-known that OA, a heterogeneous and complicated joint disorder, is characterized by the destruction of articular cartilage due to an imbalance between the biosynthesis and degradation of the ECM (1,8). Since MMPs are usually abundantly expressed in joint disorders, and the effect of these proteolytic enzymes is largely due to their ability to degrade the ECM, it is widely accepted that MMPs represent promising pharmacological targets for the treatment of OA (2,6). In particular, collagenases including MMP-1, -8 and -13 provide a suitable microenvironment for the development and progression of OA; moreover, they specifically degrade type II collagen and proteoglycans through other MMPs in the ECM of cartilage (6,9). Of the MMPs, MMP-1 is expressed ubiquitously and is found in a broad range of normal tissue cells, including chondrocytes, whereas MMP-13 is more closely linked to the degradation of type II collagen than MMP-1 or -8 and has long been regarded as a key mediator of cartilage degradation in joint disorders (6,9). In the present study, the OA microenvironment was mimicked by cultured SW1353 cells stimulated with IL-1β. As indicated in the Results, the production of MMP-1 and -13 were significantly promoted in SW1353 cells after stimulation with IL-1β, and MF inhibited MMP-13 release more than MMP-1. Conversely, MF pretreatment effectively suppressed IL-1β-stimulated MMP-1 and MMP-13 release in SW1353 cells by downregulating the overexpression of MMP-1 and -13 but not MMP-2 and -3. Thus, it is likely that treatment with MF has a significant impact on cartilage homeostasis, as it consistently inhibited the IL-1β-induced upregulation of MMP-1 and -13 at the transcriptional level in SW1353 chondrocytes.

In addition to examining the roles of MMPs in OA, elevated levels of inflammatory mediators, such as NO and PGE_2, have been previously observed in the cartilage and serum of OA patients (39,40). IL-1β-mediated overproduction of NO has also been reported to act as an important inflammatory mediator that plays a critical role in the pathogenesis of OA through inducing chondrocyte and synoviocyte deaths (41). IL-1β also stimulates the expression of COX-2 to increase the synthesis of PGE_2, which is responsible for PGE_2 and is implicated in bone resorp-

tion and joint pain in OA (42). Moreover, both NO and PGE_2 are capable of upregulating the production of MMPs and other inflammatory cytokines (43,44). Therefore, the inhibition of the production of IL-1β-induced inflammatory mediators has been shown to be useful in the treatment of OA. In the present study, we demonstrated that MMP-1 and -13 expression was suppressed by MF, and pretreatment with MF significantly decreased IL-1β-induced NO and PGE_2 production by attenuating the expression of their upstream molecules, iNOS and COX-2, on both mRNA and protein levels. These observations indicate that the expression of iNOS and COX-2 was regulated by MF at the transcriptional level and also that MF exerts anti-inflammatory effects on IL-1β-induced inflammation in chondrocytes.

It has previously been noted that NF-κB is the most important transcription factor that regulates the expression of MMPs, COX-2, and iNOS in OA (9,38). Despite the previously demonstrated function of the activator protein-1 in regulating MMP expression (45,46), data demonstrating the NF-κB-mediated induction of MMPs, in particular MMP-1 and -13, indicate that NF-κB is a potential therapeutic target in OA (37,47). Normally, NF-κB is located in the cytoplasm in an inactive form in which the heterodimer is bound to IκB inhibitor proteins. Stimulation with inflammatory molecules, such as IL-1β, leads to phosphorylation and the subsequent degradation of IκB-α, which leads to the nuclear translocation of NF-κB and the promotion of the regulation of transcription of response genes (48,49). Therefore, previous research has focused on the inhibition of MMP and inflammatory mediator expression by blocking the mobilization of NF-κB into the nucleus in chondrocytes. In addition, the high-level expression of MMPs, iNOS, and COX-2 in arthritic joints results from the activation of a tightly regulated and synchronized signaling cascade activated by IL-1β and involving the MAPKs signaling pathway (10,38). MAPKs are activated through the phosphorylation of specific tyrosine and threonine residues by the upstream kinases in response to inflammatory signals (10,11,38). In the present study, it was demonstrated that IL-1β enhances the degradation of IκB-α and enhances the translocation of NF-κB p65 from the cytoplasm to the nucleus; however, pretreatment with MF effectively attenuated the IL-1β-induced degradation of IκB-α and the nuclear translocation of NF-κB p65 in SW1353 cells. It was also observed that following stimulation of SW1353 cells with IL-1β, the activation of the JNK, p39 MAPK and ERK signaling pathways was evident. Interestingly, it was found that MF selectively inhibited IL-1β-induced p38 MAPK activation without markedly affecting ERK and JNK activation. Although other signaling pathways also play an important role in the regulation of OA-related gene expression, the results of our study suggest that the attenuation of NF-κB and p38 activity partly accounts for the inhibitory effects of MF on the gene expression of MMP-1, MMP-13, iNOS and COX-2. As previously mentioned, traditional herbal medicines have become one of the most important alternative treatments for OA. At present, the wider public is also more aware of the significance of prophylactic medications which complement traditional treatments or prevent OA. Therefore, we consider our water extract of Mori folium to be a useful nutritional supplement for OA therapy. In the future, we will further characterize the active compounds from our extract and examined its chondroprotective effect against OA and investigate the molecular mechanisms responsible in detail.

In conclusion, we have demonstrated in the present study that MF effectively suppresses the IL-1β-induced expression of MMP-1, -13, iNOS and COX-2 in SW1353 cells, all of which play a pivotal role in the progression of OA. Furthermore, we have also shown that this effect is mediated, at least in part, through the regulation of NF-κB and p38 signaling pathways. Our findings suggest that MF merits consideration as a therapeutic agent in the treatment and prevention of OA. Therefore, an in-depth study to define the bioavailability of active agent(s) in MF which are capable of exerting cartilage and chondroprotective effects *in vivo* is warranted.

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