

Tripterygium wilfordii Hook F Extract Suppresses Proinflammatory Cytokine-Induced Expression of Matrix Metalloproteinase Genes in Articular Chondrocytes by Inhibiting Activating Protein-1 and Nuclear Factor- κ B Activities

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ABSTRACT

The major pathologic manifestations of rheumatoid arthritis (RA) and osteoarthritis (OA) are joint inflammation and articular cartilage resorption by proinflammatory cytokine-stimulated matrix metalloproteinases (MMPs) and aggrecanases. The Chinese herbal remedy *Tripterygium wilfordii* Hook F (TWHF) is effective for treatment of various types of arthritis. However, mechanisms and targets of its actions are poorly understood. Anti-inflammatory activities of the extracts of this plant were previously attributed to inhibition of cyclooxygenase-2 mRNA and prostaglandin E_2 synthesis. Here, we show that in primary human femoral head osteoarthritic and normal bovine chondrocytes, TWHF partially or completely inhibited mRNA and protein expression of tumor necrosis factor- α , interleukin (IL)-1, and IL-17-inducible MMP-3 and MMP-13. This agent also inhibited cytokine-stimulated MMP-3 protein expression in human synovial fibroblasts. A dose range of 2.5 to 10 ng/ml of

TWHF was effectively inhibitory for IL-1. Pretreatment for 30 min or 1 h (but not 2–10 h) after IL-1 treatment with TWHF inhibited MMP-3 RNA induction. The inhibitory doses had no adverse effect on the viability of chondrocytes. Mechanistic studies revealed no impact on the activation of extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase mitogen-activated protein kinases. Instead, TWHF partially inhibited DNA binding capacity of cytokine-stimulated activating protein-1 (AP-1) and nuclear factor- κ B (NF- κ B) transcription factors. Therefore, besides its anti-inflammatory activity, this agent may also be effective in blocking cartilage matrix resorption by MMPs by impairing AP-1 and NF- κ B binding activities. Thus, TWHF extract contains novel inhibitors of MMP expression that may be of therapeutic potential in arthritis and other conditions associated with increased MMPs.

The collagens and aggrecan of cartilage extracellular matrix (ECM) are synthesized by chondrocytes that provide mechanical strength to joints. Rheumatoid arthritis (RA) is a chronic disease with severe joint inflammation, synovial hyperplasia, and deformation. Osteoarthritis (OA) is a prevalent and less inflammatory joint degenerative disease caused by joint overuse, obesity, aging, gender, and cartilage gene mutations (Poole, 1999). Resorption of cartilage is preceded by an initial excessive synthesis of ECM and failure of repair processes (Aigner and Dudhia, 1997) induced by an imbalance

between anabolic growth factors (transforming growth factor- β and insulin-like growth factor-I) and proinflammatory cytokines, interleukin (IL)-1, and tumor necrosis factor- α (TNF- α) (Dinarello and Moldawer, 1999). These cytokines induce matrix metalloproteinases (MMPs) that release cartilage ECM fragments, which serve as markers of arthritis (Lark et al., 1997).

MMPs include matrilysin, stromelysins, gelatinases, interstitial and neutrophil collagenases, collagenase-3 (MMP-13), and membrane-type MMPs (Westermarck and Kähäri 1999). They digest different components of the ECM during physiologic and pathologic turnover. Stromelysin-1 (MMP-3) cleaves proteoglycans, collagens, gelatins, and link protein of aggrecan. An imbalance between active MMPs and the tissue

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ABBREVIATIONS: ECM, extracellular matrix; RA, rheumatoid arthritis; OA, osteoarthritis; IL, interleukin; TNF- α , tumor necrosis factor- α ; MMP, matrix metalloproteinase; TWHF, *Tripterygium wilfordii* Hook F; COX, cyclooxygenase; AP-1, activator protein-1; NF- κ B, nuclear factor- κ B; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; EMSA, electrophoretic mobility shift assay; PEA-3, polyomavirus enhancer A binding protein-3; BSA, bovine serum albumin.

inhibitors of metalloproteinases in OA cartilage contributes to its breakdown (Dean et al., 1989). The MMPs and aggrecanase cleaves aggrecan at distinct sites (Fosang et al., 1996; Tortorella et al., 1999). Increased MMP-3 in the serum and synovium of RA patients is a marker of inflammation (Yoshihara et al., 1995). MMP-3 was localized in the superficial zone of cartilage and in the synovium of patients with OA (Okada et al., 1992). We showed that 50% of the OA patients had elevated MMP-3 mRNA in their synovium (Zafarullah et al., 1993). IL-1 increased MMP-3 expression in rabbit cartilage (Hutchinson et al., 1992). A 20-fold excess of MMP-3 over MMP-1 was reported in the synovial fluids of patients with RA (Walakovits et al., 1992). In human cartilage, MMP-3 mRNA was preferentially increased compared with MMP-1 in the presence and absence of IL-1 (Nguyen et al., 1992). MMP-13 produced by chondrocytes enhances cleavage and denaturation of type II collagen in OA cartilage (Mitchell et al., 1996; Billingham et al., 1997) and aggrecan at the MMP-specific site and a new site (Fosang et al., 1996). Its expression is increased in rheumatoid synovium (Lindy et al., 1997), OA cartilage (Reboul et al., 1996), and is further induced by IL-1 and TNF- α via c-fos mediation (Borden et al., 1996). Like other MMPs, the secreted inactive pro-MMP-13 is activated extracellularly by gelatinase A and MT1-MMP (Knäuper et al., 1996).

IL-1 and TNF- α are the main proinflammatory cytokines in joints that suppress ECM synthesis and increase MMP-mediated cartilage resorption (Dinarello and Moldawer, 1999). IL-17 is a newer proinflammatory cytokine in synovial fluid (Dinarello and Moldawer, 1999) that induces IL-1 β , IL-6, and stromelysin in chondrocytes (Shalom-Barak et al., 1998). Inhibition of IL-1 and TNF- α actions by IL-1 receptor antagonist via gene therapy and antibodies to TNF- α are beneficial in reducing the symptoms of arthritis (Feldmann et al., 1997; Oligino et al., 1999). Thus, blocking IL-1-, TNF- α -, and IL-17-induced MMP gene expression by novel physiologic and pharmacologic inhibitors (Vincenti et al., 1994; Lark et al., 1997) is an important therapeutic approach for arthritis.

The Chinese herbal remedy *Tripterygium wilfordii* Hook F (TWHF) (Lei Gong Teng or thunder god vine) has been known for thousands of years as a therapeutic agent against arthritis and autoimmune diseases (Lipsky and Tao, 1997). This agent has both immunosuppressive and anti-inflammatory activities, including inhibition of cytokine gene expression in T cells (Tao et al., 1996). The anti-inflammatory actions have been attributed to the inhibition of cyclooxygenase (COX)-2 and prostaglandin E₂ in rheumatoid fibroblasts and other cell types (Tao et al., 1998). We examined whether TWHF extract was also effective against major MMPs involved in the resorption of arthritic cartilage. Here, we show for the first time that TWHF potently inhibits proinflammatory cytokine-induced MMP-3 and MMP-13 gene expression partly by interfering with DNA binding activities of AP-1 and NF- κ B transcription factors. Furthermore, MMPs are novel targets of antiarthritic actions of TWHF.

Materials and Methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin streptomycin, Fungizone, and agarose were from Canadian Life Technologies, Inc. (Burlington, ON, Cana-

da). One hundred-millimeter plates and T-75 flasks were from Nunc (Roskilde, Denmark). IL-1 β , TNF- α , and IL-17 were obtained from R&D Systems (Minneapolis, MN). Collagenase type II was from Sigma-Aldrich (Oakville, ON, Canada). The chemiluminescence systems were from Roche Molecular Biochemicals (Laval, PQ, Canada). Zeta-probe and nitrocellulose membranes were from Bio-Rad Canada (Mississauga, ON, Canada). RNA probe labeling kits were from Promega (Madison, WI). Restriction endonucleases, T7 polymerase, and RNase inhibitor were from Amersham Pharmacia Biotech (Baie d'urfe, PQ, Canada). TWHF pills (containing 33 μ g of TWHF per pill) were obtained from Huangshi Pharmaceuticals (Hubei Province, Peoples Republic of China), dissolved in 100% ethanol, filter-sterilized, and stored at -20°C .

Primary Cultures of Bovine and Human Chondrocytes, Synovial Fibroblasts, and Treatments. Normal bovine articular cartilage was obtained from the knee joints of adult animals from a local abattoir. Human cartilage was from the femoral heads of the OA patients who underwent hip replacement surgery at Notre Dame Hospital. Chondrocytes were released by pronase (1 mg/ml) for 60 min and collagenase (Sigma type IA) digestions for 9 h in DMEM at 37°C . Cells were washed five times with phosphate-buffered saline (PBS) and grown in DMEM with 10% FCS as high-density primary monolayer cultures until confluent growth. Cells were distributed in 6-well plates, grown to confluence, washed with PBS, kept in serum-free DMEM for 24 h, TWHF extract added at the final concentrations of 2.5 and 5 ng/ml (or as indicated) 30 min before treatment with IL-1 β (10 ng/ml), TNF- α (20 ng/ml), and IL-17 (20 ng/ml) for 24 h. Human knee synovial fibroblasts at passage 13 were maintained in medium with 0.5% FCS and were subjected to the same treatments.

RNA Extraction and Northern Hybridization Analysis. Total RNA was extracted by the guanidinium isothiocyanate procedure (Chomczynski and Sacchi, 1987) and aliquots of 3 to 5 μ g analyzed by electrophoretic fractionation in 1.2% formaldehyde-agarose gels. The integrity and quantity of RNA were verified by ethidium bromide staining of the 28S and 18S ribosomal RNA bands. RNA was transferred completely onto Zeta-probe nylon membrane with a Bio-Rad Transblot in the presence of $0.5 \times$ Tris/acetate/EDTA buffer at a current of 500 mA for 12 h. Northern blots were hybridized with a human stromelysin cDNA probe generously provided by Dr. Richard Breathnach (Nantes, France). This probe, which cross-hybridizes with the bovine stromelysin RNA, was a 1.6-kilobase pair *Eco*RI cDNA fragment cloned in the plasmid pGEM-4Z (Promega Biotech) and the vector linearized with *Nar*I. A 491-base-pair reverse transcriptase-polymerase chain reaction collagenase-3 cDNA product (Shlopov et al., 1997) was cloned in pGEM-4Z, identified by DNA sequencing, and linearized with *Eco*RI. The human 28S ribosomal RNA plasmid (American Type Culture Collection, Manassas, VA) was digested with *Xba*I. All antisense RNA probes were synthesized with T7 polymerase according to the protocols of Promega Biotech and labeled to high-specific activity (1×10^8 cpm/ μ g) with [α -³²P]CTP (3000 Ci/mmol; DuPont Canada, Inc., Mississauga, ON, Canada).

Western Immunoblot Analysis. Total secreted proteins from the 2- to 3-ml conditioned medium of the bovine or human chondrocytes were concentrated 40- to 50-fold by Microcon YM-10 centrifugal filter devices (Millipore Corporation, Bedford, MA), quantified by the Bio-Rad protein assay system, and 10- to 20- μ g aliquots adjusted to 15 μ l with $4 \times$ sample buffer composed of 40 mM DTT, 37.6% sucrose, 0.032% bromophenol blue, and 8.25% SDS. Along with the prestained broad-range molecular mass standards (Bio-Rad), samples were fractionated by a 4% stacking and 10% SDS-PAGE mini-gel (Bio-Rad), and transferred to nitrocellulose membrane by electroblotting in a buffer composed of 25 mM Tris-HCl, 192 mM glycine, 0.04% SDS, and 20% ethanol at 200 mA. Membranes were rinsed with distilled water, incubated for 1 h in PBS, pH 7.4 with 5% Carnation nonfat dry milk to block nonspecific interactions, and washed five times (twice for 5 min, once for 15 min, and twice for 5 min) with PBS-0.1% Tween. They were then reacted overnight sequentially in the same

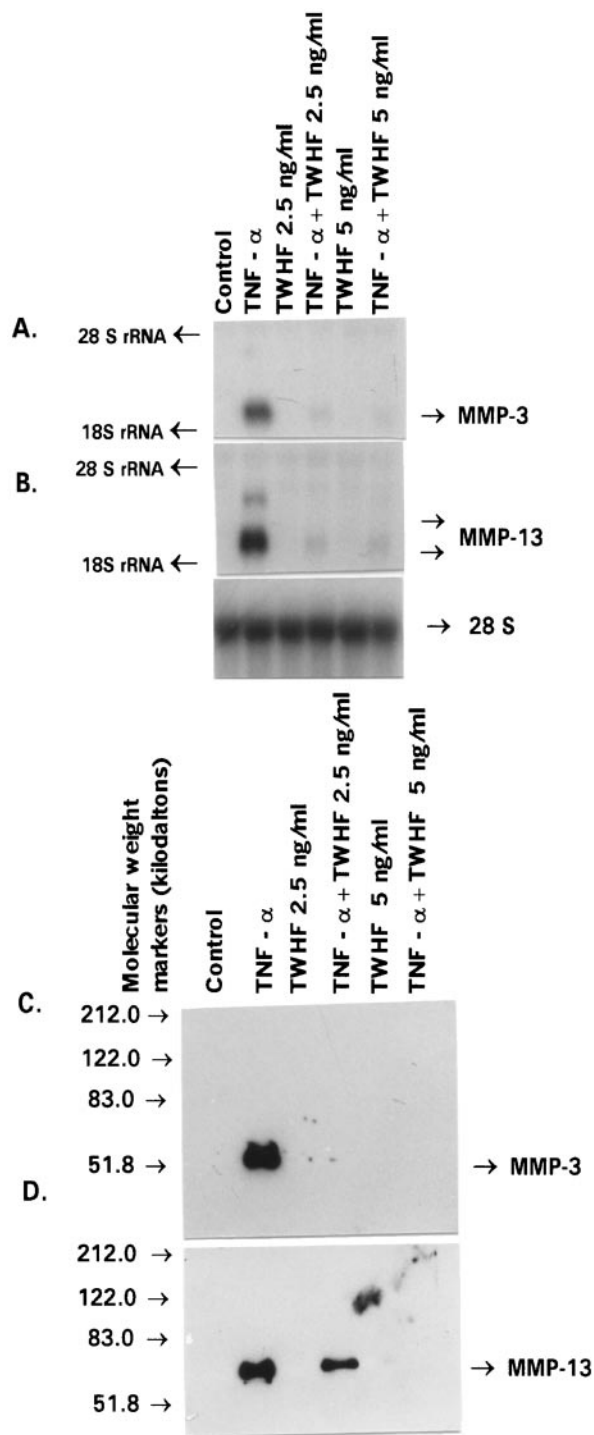


Fig. 1. Inhibition of TNF- α -induced stromelysin-1 (MMP-3) and collagenase-3 (MMP-13) gene expression by TWHF extract in human chondrocytes. Human femoral head chondrocytes from three different patients were grown to confluence, rendered quiescent by serum starvation for 24 h, pretreated with the indicated doses of TWHF for 30 min alone, or followed by the additional treatment with TNF- α (20 ng/ml) for 24 h. Control cells were treated with TNF- α and TWHF vehicles (PBS-0.1% BSA and ethanol, respectively). Results of the Northern blot analysis with MMP-3, MMP-13, and 28S RNA bands are shown in A and B. The positions of 28S and 18S ribosomal RNA bands are also indicated. In C and D, total protein (20 μ g/lane) from the conditioned media of chondrocytes subjected to the indicated treatments was subjected to SDS-PAGE, transferred to nitrocellulose, reacted with (A) anti-human MMP-3 and (B) MMP-13 antibodies, and the respective bands revealed by chemiluminescence. The positions of MMP bands relative to the known protein markers are shown by arrows.

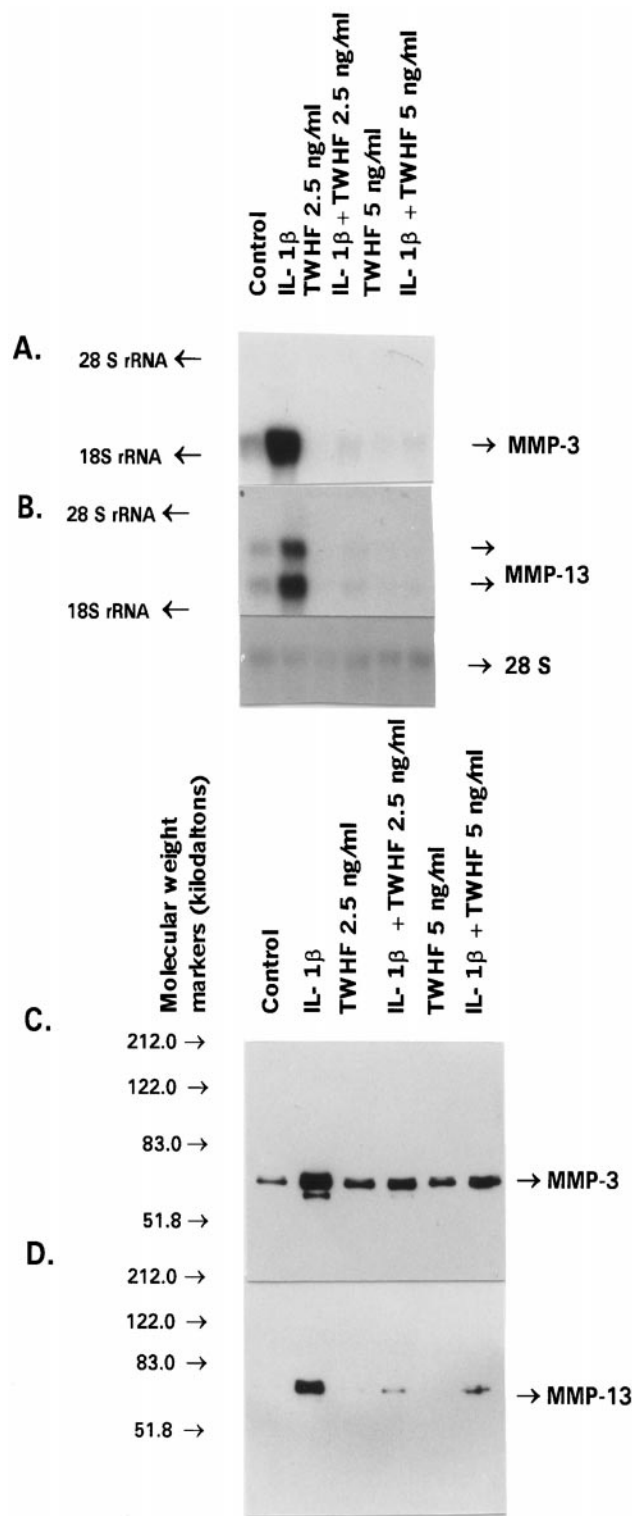


Fig. 2. Inhibition of IL-1 β -induced MMP-3 and MMP-13 RNA and protein expression by TWHF extract in human chondrocytes. Primary quiescent human chondrocytes from three different patients were pretreated with the indicated doses of TWHF for 30 min alone or followed by the additional treatment with IL-1 β (10 ng/ml) for 24 h. Control cells were treated with IL-1 β and TWHF vehicles (PBS-0.1% BSA and ethanol, respectively). Results of Northern blot analysis with the positions of MMP-3, MMP-13 and 28S RNA bands are shown (A and B). Total protein (20 μ g/lane) from the conditioned media of chondrocytes from two different patients was subjected to SDS-PAGE, transferred to nitrocellulose, reacted with anti-human MMP-3 and MMP-13 antibodies and the respective bands revealed by chemiluminescence. The positions of MMP bands relative to the protein markers are shown by arrows in C and D.

buffer at 4°C with 1 to 2 µg/ml of the mouse anti-human MMP-3 (Ab-1, Clone 55-2A4; Calbiochem, San Diego, CA) and MMP-13 antibodies (Clone 181-15A12; from Fuji Chemical Industries, Toyama, Japan). Subsequently, membranes were washed five times with PBS-0.1% Tween at room temperature, incubated with the anti-mouse secondary peroxidase-conjugated IgG (300 mU/ml), and washed seven times with PBS-0.1% Tween. For revelation of the

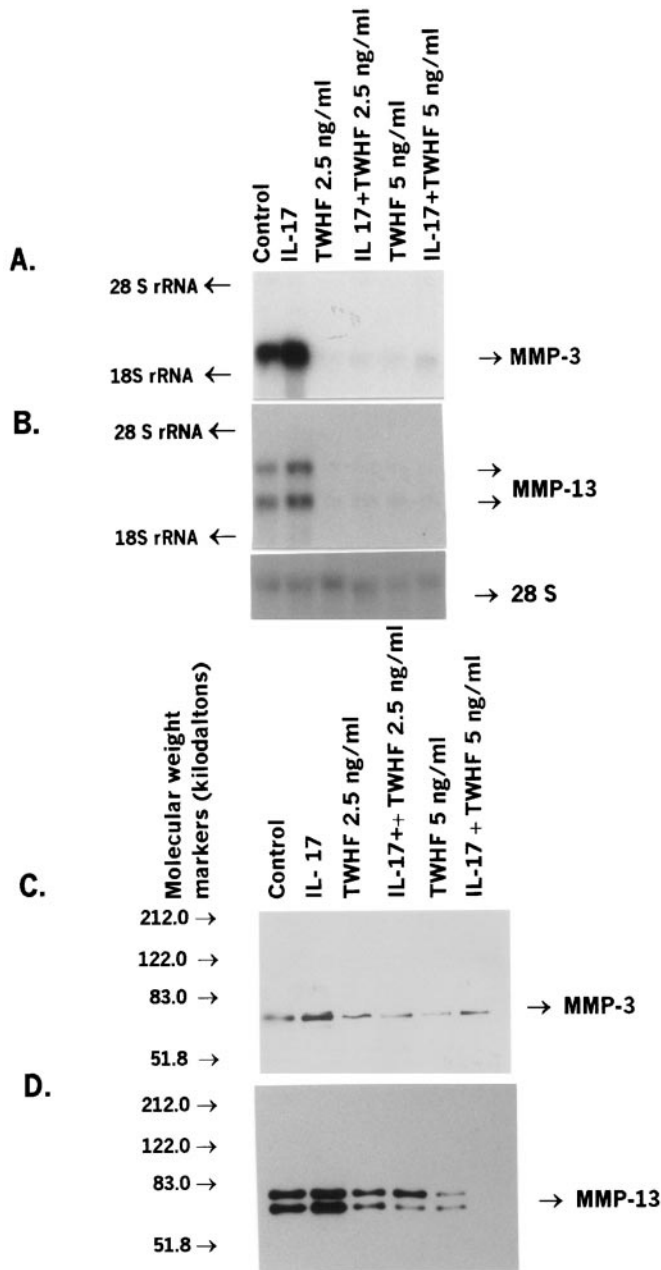


Fig. 3. Inhibition of IL-17-induced MMP-3 and MMP-13 RNA/protein expression by TWHF extract in human chondrocytes. Quiescent primary human chondrocytes from three different patients were pretreated with the indicated doses of TWHF for 30 min alone or followed by the additional treatment with IL-17 (20 ng/ml) for 24 h. Control cells were treated with IL-17 and TWHF vehicles (PBS-0.1% BSA and ethanol, respectively). The MMP-3, MMP-13, and 28S RNA bands are shown in A and B. Total protein (20 µg/lane) from the conditioned media of chondrocytes from two different patients was subjected to SDS-PAGE, transferred to nitrocellulose, reacted with anti-human MMP-3 and MMP-13 antibodies, and the respective bands revealed by chemiluminescence. The positions of MMP bands relative to the standard protein markers are shown by arrows (C and D).

MMP-3 and MMP-13 bands, membranes were incubated with 10 µl of solution A and 990 µl of solution B of the chemiluminescence detection system of Roche Molecular Biochemicals according to the manufacturer's protocols and exposed to film for 2 to 15 min. For MAPKs, chondrocytes were pretreated with TWHF (5 ng/ml) for 30 min and stimulated with TNF-α for 20 min. Cells were lysed in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 50 mM DTT, and 1% Triton X-100), and lysates were resolved on SDS-PAGE gels, transferred to nitrocellulose membranes by electroblotting and reacted with ERK, p38, and JNK phosphorylation state-specific antibodies (New England Biolabs, Beverly, MA) at concentrations suggested by the manufacturers. Proteins were visualized by horseradish peroxidase-conjugated sheep anti-rabbit IgG and the chemiluminescence detection system.

Protease Substrate Gel Electrophoresis and Zymography.

Bovine chondrocytes were subjected to different treatments for 24 h, and 30 µl of the media was treated with 1 mM of 4-aminophenylmercuric acetate, mixed with 4× sample buffer (0.25 M Tris-HCl, pH 6.8, 10% SDS, 4% sucrose, and 0.1% bromophenol blue) and applied to 10% SDS-PAGE containing 1 mg/ml α-casein (Sigma-Aldrich). After electrophoresis under nonreducing conditions, the gels were washed twice, 30 min each in 2.5% Triton X-100 (v/v), and incubated overnight in 50 mM Tris-HCl, pH 8.0, containing 5 mM CaCl₂, 1 µM ZnCl₂, and 0.02% Na₂S₂O₃ at 37°C. After staining with 0.1% Coomassie blue in water/methanol/acetic acid (5:4:1) for 10 min and destaining, caseinolytic activities (representing stromelysins) were detected by examining the zones of lysis in the respective molecular mass region and photographed. All the experiments were performed at least two (human) to three (bovine chondrocytes) times, and the results were reproducible.

Chondrocyte Viability Assays. Quiescent chondrocytes were exposed in duplicate to different doses of TWHF, incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) labeling reagent (assay of Roche Molecular Biochemicals) for 4 h, resulting in purple formazan crystals (produced as a result of metabolic activity of the cells) solubilized by overnight incubation and absorbance ($A_{550} - A_{690}$ nm) measured by spectrophotometry.

Electrophoretic Mobility Shift Assay (EMSA). For extraction of nuclear proteins after exposure to TWHF and cytokines, chondrocytes were washed with cold PBS and scraped with 1.5 ml of cold PBS. Cells were centrifuged for 10 s and resuspended in 400 µl of cold buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Cells were allowed to swell on ice for 10 min and were then vortexed for 10 s. Samples were centrifuged for 10 s and the supernatant discarded. The nuclear pellets were resuspended in 50 µl of cold buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) and kept on ice for 30 min for high salt extraction. Cellular debris was removed by centrifugation for 20 min and supernatant fraction (nuclear extract) stored at -70°C. EMSAs were performed using gel shift assay core system as described in Promega protocols. Nuclear proteins (2 µg) were mixed with ³²P-end-labeled, double-stranded AP-1 or NF-κB binding consensus oligonucleotides at room temperature for 20 min. The DNA-protein complex formed was separated from free oligonucleotides on 4.5% native polyacrylamide gel.

Results

To investigate the mechanism of antiarthritic actions of TWHF and to study its impact on human chondrocyte MMPs, femoral head primary chondrocytes from three OA patients were cultured to confluence, made quiescent, pretreated with TWHF for 30 min, and stimulated with TNF-α for 24 h. Total RNA was probed sequentially with the human MMP-3,

MMP-13, and 28S probes and protein levels in the conditioned medium by Western immunoblot analysis with the specific antibodies. TNF- α stimulated accumulation of MMP RNA and protein without any major impact on the control 28S rRNA. TWHF (but not its ethanol vehicle, not shown) dose dependently inhibited TNF- α -induced MMP-3 and MMP-13 mRNA (Fig. 1, A and B) and protein expression (C and D). The double MMP-3 protein bands most likely correspond to inactive (upper) and activated (lower) forms of the enzyme. A partial inhibition of MMP-13 protein was seen at 2.5 ng/ml, and a complete suppression was observed at 5 ng/ml of TWHF (Fig. 1). Thus, TWHF effectively and reproducibly inhibited TNF- α -stimulated MMP-3 and MMP-13 gene expression in human chondrocytes.

IL-1 is another cytokine of major importance in joint inflammation and cartilage resorption (Joosten et al., 1999). To

examine the impact of TWHF on IL-1 β -induced MMP-3 and MMP-13, quiescent human femoral head primary articular chondrocytes from three OA patients were pretreated with TWHF for 30 min and stimulated with IL-1 β for 24 h. Total RNA was hybridized sequentially with human MMP-3, MMP-13, and 28S probes. The conditioned media of the two different patients were subjected to Western immunoblot analysis with the specific antibodies sequentially. IL-1 β increased expression of MMP-3 and MMP-13 RNA and protein whose levels were partially (Fig. 2, C and D) or completely (Fig. 2, A and B) inhibited by TWHF. Therefore, TWHF reproducibly inhibited IL-1 β -stimulated MMP-3 and MMP-13 mRNA and protein expression in human chondrocytes.

IL-17 is another important proinflammatory cytokine in arthritis that induces IL-1 β , IL-6, NF- κ B, and MMP-3 in

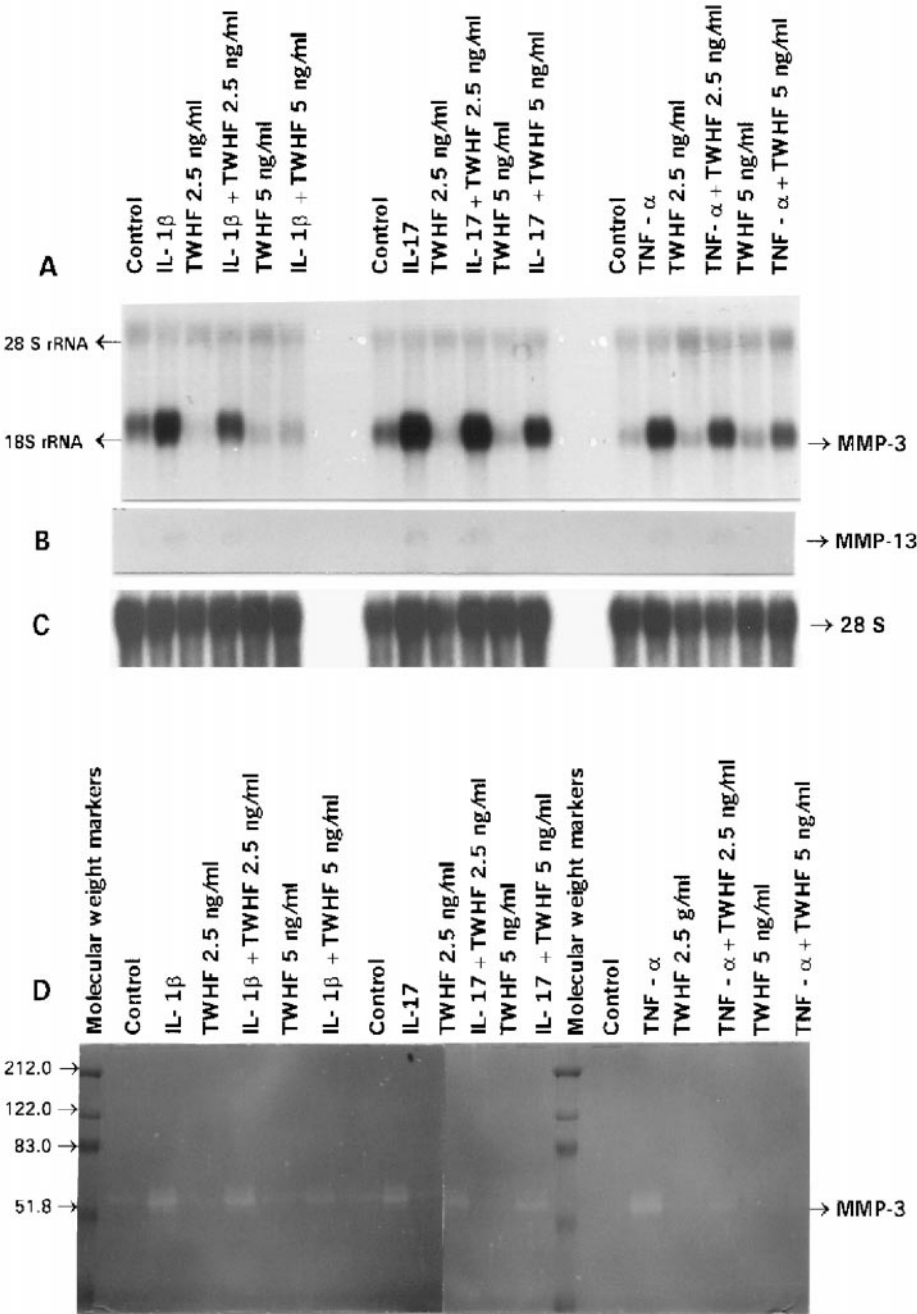


Fig. 4. Inhibition of proinflammatory cytokine-stimulated MMP-3 and MMP-13 expression in normal bovine chondrocytes. Cells were made quiescent for 24 h, pretreated with the indicated doses of TWHF for 30 min alone, or followed by the additional treatment with IL-1 β , IL-17, and TNF- α for 24 h. Control cells were treated with cytokine and TWHF vehicles (PBS-0.1% BSA and ethanol, respectively). Northern blot of RNA was hybridized with (A) human MMP-3, (B) MMP-13, and (C) 28S probes and resulting bands are shown. The positions of 28S and 18S ribosomal RNA bands after ethidium bromide staining are also indicated. In D, MMP-3 activity by zymography of the conditioned medium on casein substrate in the gel is shown.

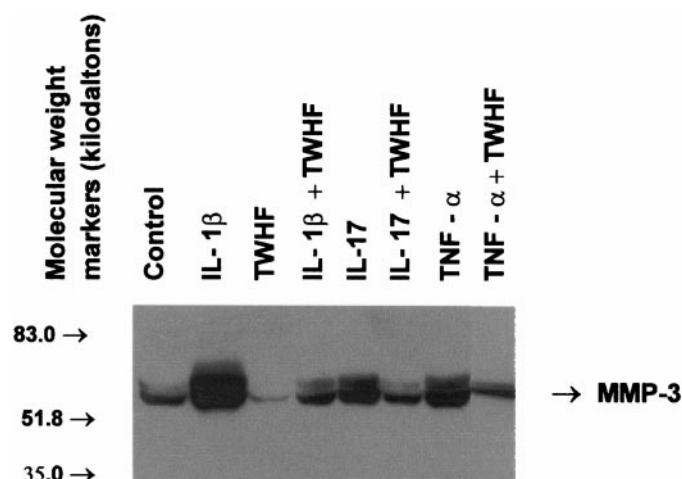


Fig. 5. Repression of proinflammatory cytokine-stimulated MMP-3 protein expression in human synovial fibroblasts. Confluent cells were made quiescent for 24 h in serum-deficient medium, pretreated with TWHF (5 ng/ml) for 30 min alone, or stimulated by additional treatment with IL-1 β , IL-17, and TNF- α for 24 h. Control cells were treated with cytokine and TWHF vehicles (PBS-0.1% BSA and ethanol, respectively). The result of Western blot analysis with human MMP-3 antibody is shown.

articular chondrocytes (Shalom-Barak et al., 1998). To examine the impact of TWHF on MMP-3 and MMP-13 RNA and protein induction by IL-17, primary human chondrocytes from two different OA patients were pretreated with TWHF for 30 min and then stimulated with IL-17 for 24 h. Total RNA was hybridized sequentially with human MMP-3, MMP-13, and 28S probes. Basal MMP-3 and MMP-13 RNA/protein levels were considerably elevated in these patients; nevertheless, a moderate induction of MMP-3 and MMP-13 was observed (Fig. 3). TWHF effectively inhibited IL-17-induced MMP-3 and MMP-13 mRNAs (Fig. 3, A and B) and a partial inhibition of the basal and induced respective pro-

teins in the conditioned medium was observed by Western blot analysis (Fig. 3, C and D). The double bands most probably correspond to inactive (upper) and activated (lower) forms of the enzymes. Thus, TWHF reproducibly repressed IL-17-stimulated MMP-3 and MMP-13 gene expression in human articular chondrocytes.

To examine the impact of cytokines and TWHF on MMP gene expression in normal chondrocytes, confluent primary bovine chondrocytes were rendered quiescent in serum-free medium for 24 h; stimulated with recombinant human IL-1 β , TNF- α , and IL-17; or pretreated for 30 min with 2.5 and 5 ng/ml of TWHF alone, followed by an additional treatment with the cytokines for 24 h. Cells were harvested for RNA analysis and conditioned medium for MMP enzyme activity analysis by zymography. The three cytokines induced MMP-3 RNA, and extracts of TWHF dose dependently inhibited its cytokine-stimulated expression (Fig. 4A). The maximal inhibition was seen with 5 ng/ml of TWHF. The observed induction and inhibition at the RNA level was also seen by zymography on the casein substrate, a technique that measures the stromelysin enzyme activity (Fig. 4D). MMP-13 RNA was also analyzed by using a human probe. A weak band corresponding to the bovine MMP-13 RNA was dose dependently inhibited by TWHF similarly to that of MMP-3 RNA (Fig. 4B). The control 28S rRNA levels were not affected by the treatments (Fig. 4C). The impact of TWHF on MMP-13 protein could not be assessed due to lack of bovine MMP-13 antibody.

To investigate the impact of TWHF on other cell types, human synovial fibroblasts (which also contribute to cartilage breakdown) were also pretreated with TWHF and stimulated with cytokines. Analysis of the secreted medium from these cells by immunoblotting demonstrated that MMP-3 was increased by all three cytokines; IL-1 was the most potent inducer. As in chondrocytes, TWHF anal-

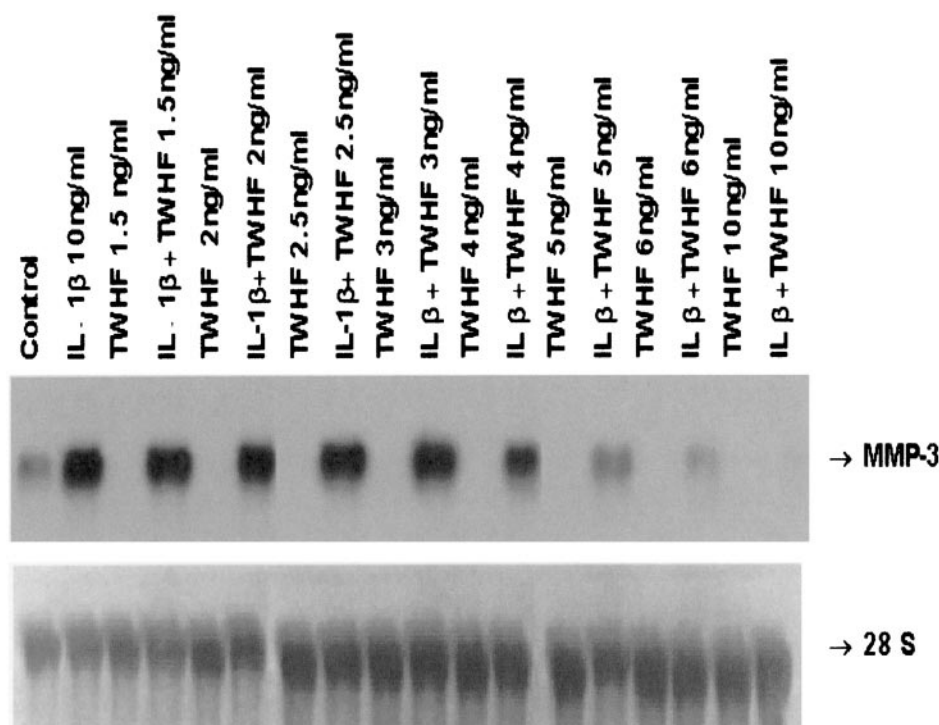


Fig. 6. Dose-dependent inhibition of interleukin β -stimulated MMP-3 RNA expression in normal bovine chondrocytes. Quiescent chondrocytes, pretreated with the indicated doses of TWHF for 30 min alone or followed by the additional treatment with IL-1 β for 24 h. Northern blot of RNA hybridized with the human MMP-3 and 28S probes is shown.

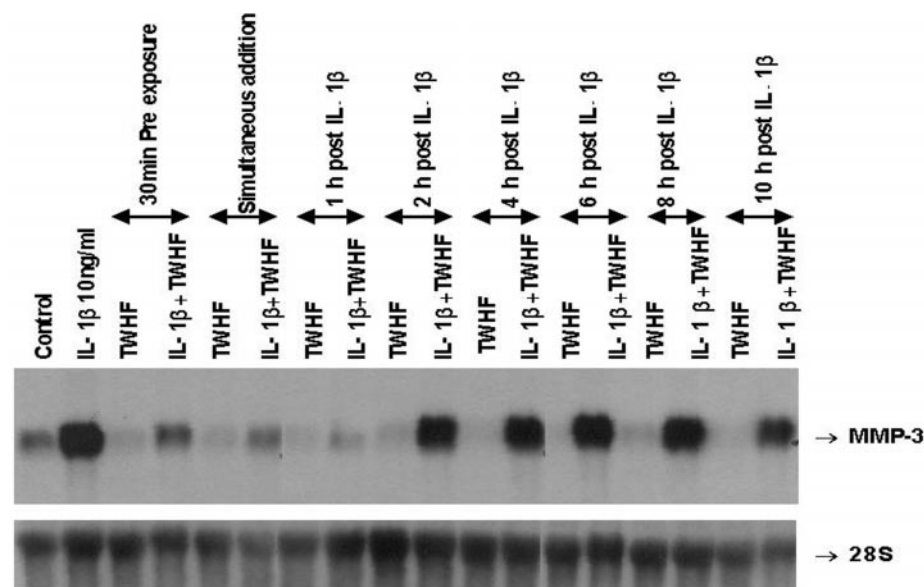


Fig. 7. Impact of pretreatment and delayed addition of TWHF on interleukin β -stimulated MMP-3 RNA expression in bovine chondrocytes. Quiescent chondrocytes were exposed to TWHF (5 ng/ml) at the indicated time points simultaneously, before, or after stimulation with IL-1 (10 ng/ml) for 24 h. Northern blot of RNA hybridized with the human MMP-3 and 28S probes is shown.

ogously inhibited MMP-3 induction, reducing it to basal levels (Fig. 5). Experiments with a chondrosarcoma cell line also showed similar MMP inhibition (results not shown). Thus, TWHF is able to inhibit MMP gene expression in diverse cell types.

Dose-response studies with TWHF and IL-1 stimulation demonstrated that contrary to inhibitory dose of 2.5 ng/ml for human OA chondrocytes, in bovine chondrocytes, dose-dependent inhibition of MMP-3 RNA started at the dose of 4 ng/ml (Fig. 6). Time course experiments showed that 30-min pretreatment with TWHF, simultaneous addition of TWHF and IL-1, as well as TWHF addition 1 h after IL-1 stimulation, resulted in potent down-regulation of the MMP-3 RNA expression. Addition of TWHF between 2 to 10 h after IL-1 did not suppress MMP-3 induction. The control 28S RNA levels were not affected by the treatments (Fig. 7). To examine if TWHF affected cell viability, bovine chondrocytes were exposed to different doses of TWHF for 24 h in two separate experiments and metabolic activity of viable cells measured by the MTT assay. TWHF extract had no adverse effect on the viability of chondrocytes and was even slightly stimulatory for these cells (Fig. 8)

Potential mechanisms for the repression of MMP expression by TWHF were studied. Because MAPKs are considered important mediators of proinflammatory cytokines (Geng et al., 1996), we evaluated the effect of TWHF on the activation of MAPKs in chondrocytes. In agreement with other studies, exposure of chondrocytes to TNF- α rapidly (within 5–20 min) stimulated phosphorylation of ERK, p38, and JNK subclasses of MAPKs. Pretreatment with TWHF for 30 min did not affect phosphorylation of any of these MAPKs (Fig. 9). AP-1 transcription factor binding sites are found in the promoters of MMP-3 and MMP-13 genes, and NF- κ B can regulate MMP expression (Pendás et al., 1997; Bond et al., 1999). To further investigate the impact of TWHF on the activity of these factors, EMSA with nuclear extracts from treated human chondrocytes revealed that TWHF partially or fully suppressed cytokine-stimulated cognate site binding activities of AP-1 and NF- κ B factors (Fig. 10). Thus, TWHF may repress MMP expression in part by interfering with these factors.

Discussion

Arthritis is a major disease, and due to limited efficacy of nonsteroidal anti-inflammatory drugs, hip and knee replacement surgery is a common intervention performed for relieving the disabling symptoms of the disease. MMP-3 and MMP-13 are the two main enzymes involved in the erosion of cartilage extracellular matrix in the patients with arthritis. We have shown here for the first time that extract of the Chinese herbal remedy, TWHF, can effectively suppress proinflammatory cytokine-induced MMP RNA and protein expression in human OA, normal bovine chondrocytes, and human synovial fibroblasts partly by interfering with DNA binding ability of AP-1 and NF- κ B transcription factors. MMPs are targets of TWHF, and their observed inhibition may be one of the mechanisms for its beneficial effects in the patients with arthritis. Therefore, besides its known antiin-

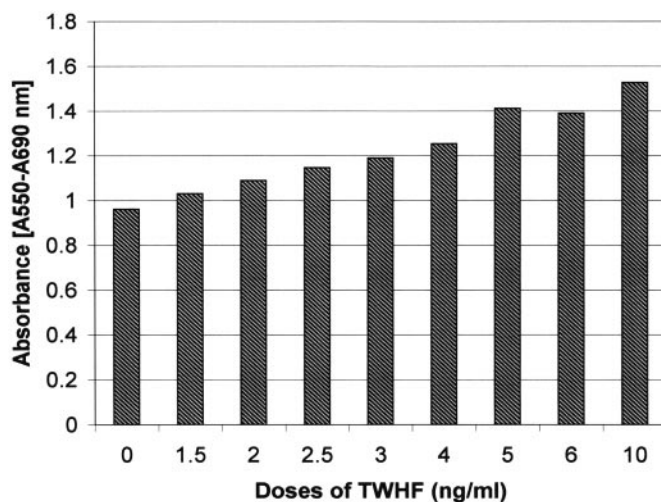


Fig. 8. Effect of TWHF extract on bovine chondrocyte viability. Quiescent bovine chondrocytes were either treated with ethanol vehicle (0 ng/ml) or exposed to the indicated doses of TWHF for 24 h, incubated with the MTT reagent, precipitate solubilized, and subjected to spectrophotometry. The bar graph with the mean absorbance values of two separate experiments is shown.

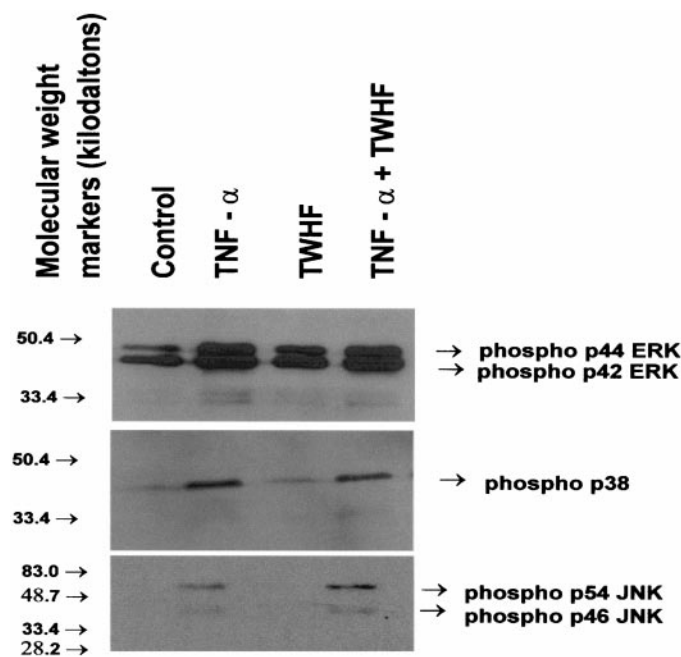


Fig. 9. Stimulation of MAPK activation by $\text{TNF-}\alpha$ and impact of TWHF. Human chondrocytes were pretreated with 5 ng/ml of TWHF for 30 min and stimulated with $\text{TNF-}\alpha$ for 20 min. Cell extracts (10 $\mu\text{g/lane}$) were analyzed for ERK1/2, p38, and JNK phosphorylation by Western blotting. The respective bands detected by chemiluminescence and identified by their sizes are shown.

flammatory activity, TWHF may also be a cartilage protective agent that deserves additional research.

Human cartilage and released chondrocytes from patients with arthritis have increased levels of MMP RNA and protein (Nguyen et al., 1992). An increased basal level of MMP-3 and MMP-13 in some patients is possibly associated with the degree of their arthritis. Variable low and high levels of MMP-13 have been observed in human OA chondrocytes (Tardif et al., 1999). The proinflammatory cytokines are increased in the joints of patients with arthritis (Dinarello and Moldawer, 1999). In agreement with previous studies, chondrocytes ex vivo can be stimulated to express increased MMP-3 and MMP-13 analogously to the in vivo situation (Nguyen et al., 1992; Borden et al., 1996). Experiments with bovine cartilage demonstrated that chondrocytes from this important ex vivo model system can also be stimulated with cytokines to express MMPs, and due to the limited availability of human cartilage constitute a good system for screening antiarthritic drugs such as TWHF. The suppression by TWHF was reproducible in bovine chondrocytes and in cells from numerous patients with OA of the femoral head. However, higher doses of TWHF were required to inhibit MMPs in normal bovine chondrocytes (5 ng/ml), compared with those for human arthritic chondrocytes (2.5 ng/ml). The response seems to be specific because 28S rRNA control RNAs were not suppressed by TWHF. Recent work by specific inhibition of IL-1 with IL-1 receptor antagonist and that of $\text{TNF-}\alpha$ by a soluble binding protein revealed that IL-1 is important for cartilage destruction and $\text{TNF-}\alpha$ stimulates joint inflammation (Joosten et al., 1999). The fact that TWHF extract inhibits the actions of both cytokines in the main cell types affected by arthritis (chondrocytes and synovial fibroblasts) suggests that the major aspects of pathogenesis of arthritis could be intercepted by the extract.

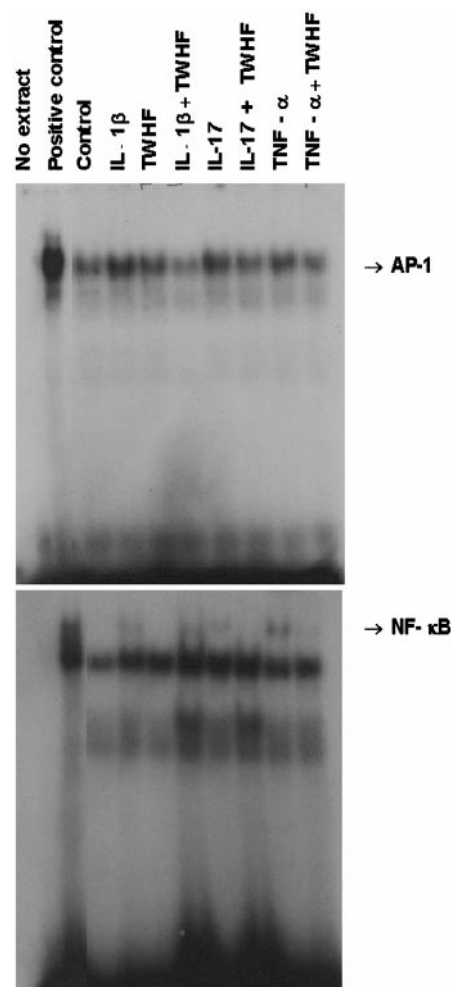


Fig. 10. Inhibition of proinflammatory cytokine-enhanced AP-1 and NF- κB DNA-binding activity by TWHF. Primary human chondrocytes were pretreated with ethanol (TWHF vehicle; control) or TWHF (5 ng/ml) for 30 min and then exposed to IL-1, IL-17, or $\text{TNF-}\alpha$ for 30 min. Nuclear extracts were prepared and DNA-binding activities for AP-1 (A) and NF- κB (B) were analyzed by EMSA using oligonucleotides containing the respective consensus binding sites. No extract lane indicates ^{32}P -labeled AP-1 or NF- κB -binding oligonucleotide without nuclear extract. Positive control lane represents nuclear extracts from HeLa cells (from Promega) reacted with the respective oligonucleotides. The positions of DNA-protein complexes are shown by arrows.

Although this plant has at least 70 active ingredients (Lipsky and Tao, 1997), triptolide is the major active component that reduces collagen-induced arthritis in rats (Gu and Brandwein, 1998). An ethyl acetate extract (called Texas ethyl acetate or TEA) has been used to demonstrate the inhibition of COX-2 (Tao et al., 1998). Another active ingredient of TWHF is multiglycosides (Maekawa et al., 1999). In our study, we have used an ethanol extract that may be similar to the previously reported ethyl acetate extract. Ethanol itself does not inhibit MMP expression (results not shown). However, further work is needed to identify precisely the component responsible for MMP inhibition that is most likely to be triptolide. Our preliminary results support this notion.

The mechanisms of action of TWHF extracts are not known. Previous work had identified cyclooxygenase-2 as a target (Tao et al., 1998). Our work has characterized MMPs as novel targets of its therapeutic actions. The fact that both

mRNA and protein are inhibited in a similar fashion suggests that the inhibition of MMPs is at the transcriptional or pretranscriptional levels. IL-1, TNF- α , and IL-17 have been shown to induce the activation of three subclasses of MAPKs, such as ERKs, JNKs/stress-activated protein kinases by a cascade of phosphorylation steps (Geng et al., 1996). Our experiments indicated, however, that although all three subclasses of MAPKs are activated in response to TNF- α , TWHF extract did not inhibit their phosphorylation. Because MMP induction by all the three studied cytokines is inhibited, a common step leading to MMP stimulation may be impaired by TWHF.

Our work identified interference with cognate binding site ability of AP-1 and NF- κ B transcription factors as possible mechanisms of TWHF action. AP-1 factor binds directly with the promoter sequences of the MMP genes. A comparison of MMP promoters revealed TATA, AP-1, and polyomavirus enhancer-A binding protein-3 (PEA-3) in different combinations. The human MMP-3 promoter has AP-1 and PEA-3 elements (Westermarck and Kähäri, 1999). The human collagenase-3 promoter has TATA, AP-1, PEA-3, and OSE-2 sequences (Pendás et al., 1997). Although the NF- κ B binding site was previously localized only in the promoter of MMP-9, recent work has shown that this factor is also a very important component in the regulation of MMP-1 and MMP-3 genes (Vincenti et al., 1998; Bond et al., 1999). A recent report suggested that IL-1-induced COX-2 expression was inhibited via prevention of the NF- κ B (but not AP-1) factor binding to its target DNA by TWHF extracts in synovial fibroblasts (Maekawa et al., 1999). Thus, inhibition of MMP-3 and MMP-13 gene expression partly involves prevention of AP-1 and NF- κ B binding to the respective promoters. This coincides well with our observation that the agent is inhibitory during earlier blockade of transcription by pretreatment or within 1 h after cytokine stimulation. By preventing this binding, TWHF may also interfere with AP-1/NF- κ B interaction, consequently reducing the activity of AP-1. COX-1 and COX-2 overexpression by stable transfection has recently been shown to induce membrane type-MMP1, which activates MMP-2 (Takahashi et al., 1999) suggesting possible interaction between COX and MMPs. Thus, inhibition of COX by TWHF (Tao et al., 1998) may also result in the down-regulation of MMP-3 and MMP-13 expression.

Current arthritis treatments include nonsteroidal anti-inflammatory drugs, COX-2 inhibitors, and glucocorticoids. Selective synthetic inhibitors of collagenase-3 have been developed that prevent cartilage damage (Billinghurst et al., 1997). The results of earlier studies and these studies show that, due to its anti-inflammatory (Lipsky and Tao, 1997) and anticatabolic activities, TWHF could be a source of novel antiarthritic drugs. Almost no adverse effects on the in vitro chondrocyte viability can be an added benefit. Such viability and cytotoxicity studies need to be extended to in vivo models. Recent clinical trials have demonstrated the use of topical applications of TWHF to be beneficial for RA joints (Cibere et al., 2000). Besides arthritis, MMPs are also important modulators of angiogenesis and metastasis of cancer cells, and their inhibition is a major goal in cancer treatment (Westermarck and Kähäri, 1999). The reported antimetastatic and antiangiogenic activities of TWHF extracts in vivo (Ushiro et al., 1997) may be due to their ability to inhibit MMPs, as demonstrated in our study.

In summary, we have demonstrated that proinflammatory cytokines, IL-1, IL-17, and TNF- α induce MMP-3 and MMP-13 RNA and protein in primary human and bovine chondrocytes. The extracts of the Chinese herbal remedy, TWHF, possibly triptolide, reproducibly suppress this induction in part by interfering with AP-1 and NF- κ B DNA binding activities. This agent could serve as a source and template for novel antiarthritic and cartilage protective drugs. MMP inhibition may be an important mechanism for the observed beneficial effects of TWHF in patients with arthritis. Suppression of MMPs by TWHF may also be useful in the prevention of metastasis by cancer cells and plaque rupture by MMPs in cardiovascular diseases.

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