대한침구학회지 제23권 제2호(2006년 4월)
The Journal of Korean Acupuncture & Moxibustion Society

Original Article

NF-kB and AP-1-regulatory Mechanism of Buthus Martensi Karsch Herbal Acupuncture Solution on Inflammatory Cytokine-induced Human Chondrocytes Dysfunction

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Abstract

Objectives : Human chondrocytes co-treated with Buthus martensi Karsch herbal acupuncture solution(BMK-HAS) extract produced significantly less NO compared with chondrocytes stimulated with IL-1β alone

Methods : Activation and translocation of and NF-kB DNA binding activity were determined by Western blotting and specific enzyme-linked immunosorbent assay.

Results : The inhibition of NO production correlated with the suppression of induction and expression of nuclear factor-kB (NF-kB) and activation protein-1 (AP-1)-dependent gene. BMK-HAS inhibited the activation and translocation of NF-kB to the nucleus, indicating that BMK-HAS inhibits the IL-1β -induced production of NO in human chondrocytes by interfering with the activation of NF-kB through a novel mechanism. In addition, BMK-HAS reduced prostaglandin E2 (PGE2)production in mouse peritoneal macrophages stimulated with lipopolysaccharide, whereas no influence on the activity of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) or cyclooxygenase-1 (COX-1) was observed. My data, therefore, suggest that BMK-HAS may be a therapeutically effective inhibitor of IL-1β-induced inflammatory effects that are dependent on NF-kB activation in human OA chondrocytes.

· **Acceptance** : 2006. 3. 2. · **Adjustment** : 2006. 3. 18. · **Adoption** : 2006. 3. 18.
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Conclusion : The results indicate that BMK-HAS exerts anti-inflammatory effects related to the inhibition of neutrophil functions and of NO and PGE2 production, which could be due to a decreased expression of iNOS and COX-2 through the transcription factors NF-kB and AP-1.

Key words : Buthus martensi Karsch (BMK), herbal acupuncture solution (HAS), IL-1β, chondrocytes, nitric oxide (NO), inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), cytokine, OA.

Ⅰ. Introduction

Recently, BMK-HAS, as a traditional Korean immunosuppressive agent, has been widely used in the treatment of some immune-related diseases, especially osteoarthritis and satisfactory results are obtained[1], as also described for osteoporosis and bone resorption, according to the traditional Chinese and Korean literature[2].

The effect of scorpion BMK-HAS venom on plasma extravasation and paw withdrawal latency to radiant heat was reported[3]. BMK-HAS venom by subcutaneous injection under the surface of the rat hind paw caused dose-dependant increased plasma extravasation. BMK-HAS venom may provide a valuable resource for controling a number of inflammatory diseases and identifying potential immunomodulating, anti-infarction, anti-allergic, analgesic and anti-inflammatory effects.

Among the different scorpion species, Buthus martensi Karsch herbal acupuncture solution (BMK-HAS), a widely distributed scorpion species in Asia, has received a lot of attention. Indeed, over the past decade, more than 70 different peptides, toxins or homologues have been isolated[4]. Nociceptive afferent fibers could be activated to induce excitatory amino acid release from spinal dorsal horn by nociceptive factors BMK-HAS-I, but the delayed release of GABA was attributed to the modulating role of some antinociceptive components in the venom[3]. In mammalian cells, regulation of iNOS expression is predominantly governed by the ubiquitously expressed transcription factor nuclear factor-kB (NF-kB) and activation protein-1 (AP-1), which are required for the inducible expression of genes associated with inflammatory responses[5].

Although treatment with BMK-HAS could inhibit the onset and development of arthritis and the immune responses to collagen, without changing the severity when the disease was established, little is still known about the mode of action of this toxical medication on osteoarthritis. The need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study.

Ⅱ. Experiments

1. Materials

Culture flasks and dishes were obtained from Nunc (Roskilde, Denmark). Media and sera for cell culture were purchased from Jeil Biotech Services (Daegu, Korea). Tissue culture media and reagents, Fetal bovine serum (FBS) was from Gibco (Chagrin Falls, OH, USA). The mRNA isolation kit and DNA labelling kit were obtained from Takara Co. (Osaka, Japan) and Promega (Madison, WI, USA). Recombinant human IL-1β was obtained from Genzyme Corp. (Cambridge, MA, USA). All other chemicals and biochemicals were of analytical grade and were purchased from Sigma Chem. Co. (St. Louis, MO, USA) or

Boehringer Mannheim Biochemicals (Seoul, Korea).

The aqueous extracts of BMK-HAS (B-12-13), which was massproduced as for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University College of Oriental Medicine (Kyungju, Korea). BMK-HAS for application in cell culture was suspended in normal saline at a concentration of 1 ㎎/㎖.

2. Preparation of articular chondrocytes

Macroscopically normal cartilage samples were obtained from OA patients (5 samples) at the time of total joint replacement. The Knee cartilage was procured according to the guidelines of the Cooperative Human Tissue Network for the use of discarded human tissue and with the approval of the Institutional Review Board of Dongguk University. Chondrocytes were prepared from cartilage samples by enzymatic digestion essentially as previously described. Isolated chondrocytes were plated at a density of 1 -106/㎖ in 60-mm tissue culture dishes (Corning, NY, USA) in Ham's F-12 medium: Dulbecco's modified Eagle's medium (1:1), supplemented with L-glutamine, penicillin-streptomycin, Fungizone, and 10% fetal bovine serum (complete medium), and allowed to adhere to the tissue culture plates for 72 hours at 37℃ with 5% CO_2 and 95% air.

3. Treatment of chondrocytes with IL-1β and BMK-HAS

Human chondrocytes (1x106/ml) were plated in 6-well plates and serum-starved for 12 hours/ overnight. The medium was replaced with fresh medium containing recombinant human IL-1β (2 ng/ml), and chondrocytes were incubated for 24 hours in a tissue culture incubator at 37℃ and 5% CO_2. This concentration of IL-1β was chosen based on pilot experiments performed to determine the lowest concentration of IL-1β that would induce the maximum production of NO by OA chondrocytes in vitro. To study the dose-dependence of the effect of BMK-HAS on IL-1β-induced iNOS expression, chondrocytes were pretreated for 30 minutes with varying concentrations of BMK-HAS (1-10 ㎍/ml) before the addition of IL-1β. Controls consisted of chondrocytes incubated in the culture medium without BMK-HAS or IL-1β.

4. Western blot analysis

After the treatments, chondrocytes lysate were prepared as described previously[6] and used for Western blot analysis. For studies involving NF-kB, nuclear and cytosolic fractions were prepared. Protein content of the lysates was determined using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, USA), and 25 g of total protein/lane was resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies for NF-kB/p65, iNOS, eNOS, or nNOS were either rabbit polyclonal antibodies or mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were blocked with 5% nonfat dried milk or 2% bovine serum albumin in Tris-buffered saline (TBS) containing0.05% Tween 20 (TBST). Primary antibodies were diluted in TBST and incubated with the blots for >2 hours at room temperature, washed with fresh TBST, and incubated with a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) in blocking buffer for 1 hour at room temperature. After washing, the protein bands were visualized with the ECL detection kit (Amersham, Arlington Heights, IL) and Kodak Bio-Max film. Blots shown are representative of 3 independent experiments using chondrocytes from independent cartilage samples. To detect -actin protein, the membranes incubated with the mouse monoclonal -actin antibody (Chemicon, USA). Detection was performed using a secondary horseradish peroxidase-linked anty-mouse antibody and the ECL

chemiluminescence system (Amersham, USA).

5. NF-kB DNA binding activity assay

Cellular levels of active NF-kB/p65 in the nuclei of human chondrocytes treated with IL-1β and BMK-HAS were determined with a highly sensitive and specific colorimetric method, using a commercially available reagent kit according to the instructions of the immunosorbent assay reader at 450㎚ with a reference wavelength of 655 ㎚. Wild-type and mutated NF-kB oligonucleotides supplied in the kit were used as specificity controls and were added to some wells prior to the addition of chondrocyte lysate.

6. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Poly(A)+ RNA was isolated from OA chondrocytes using oligo (dT) affinity chromatography according to Oligotex kit instructions (Quiagen Korea Co., Seoul, Korea). Total cytoplasmic RNA was prepared from human chondrocytes using a commercially available kit according to the instructions of the manufacturer. Author used real-time RT-PCR with internal fluorescent hybridization probes in an ABI Prism 7700 detection system (ABI/Perkin Elmer Biosystems, Foster City, CA, USA), which allows the sensitive and specific quantification of targeted mRNA transcripts. The target-specific RTprimer (5-CTCTggTCAAAC-3), PCR primers (forward 5-AgCgggATgACTTTCCAAgA-3; reverse 5-ATAATggACCCCAggCAAgATT-3, and the TaqMan Probe for iNOS(6FAM-CCATAAggCCAAAgggATTTTAACTTgCAg-TAMRA) were designed using the Primer Express software (ABI/Perkin Elmer Biosystems). The probe was labeled with 5 carbofluorescein (FAM) at the 5 end and with TAMRA at the 3 end (ABI/Perkin Elmer Biosystems). The degradation of the probe during PCR resulted in increased fluorescence of the probe, which specifically annealed between the forward and reverse primer sites, and allowed the detection of the PCR product by monitoring the increase in fluorescence of the reporter dye.

To quantify the expression of iNOS, single-stranded complementary DNA (cDNA) was synthesized using 100 ng of total RNA prepared from OA chondrocytes as described above. The RNA was mixed with 10 M of RT primer, Moloney murine leukemia virus reverse transcriptase, RNase inhibitor, 0.1 M DTT, buffer, and dNTPs using a commercially available kit according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). The quantitative PCR reactions were set up using 5ℓ of cDNA and 20ℓ TaqMan Universal PCR Master mix (Perkin Elmer Biosystems), which contains reaction buffer, optimal amounts of AmpliTaq Gold DNA polymerase, AmpErase UNG (which protects against amplicon carryover), and dNTPs with dUTP. Concentrations of primers and probe used were optimized in pilot studies to allow accurate quantitation of the target transcript. The PCR conditions were 1 cycle at 50℃ for 2 minutes (for optimal AmpErase UNG enzyme activity), 1 cycle at 95℃ for 10 minutes (to activate AmpliTaq Gold DNA polymerase), followed by 40 cycles (95℃ for 15 seconds, 60℃ for 1 minute). The cycle threshold value (CT) for the samples analyzed was compared with the CT value of the known amounts of standard iNOS cDNA constructed and amplified simultaneously. To ensure lack of DNA contamination in the RNA samples, a tube of sample without RT was included as a control. Expression of iNOS was corrected to the expression of ribosomal 18S gene (R18) and the results were expressed as copies of iNOS/1010 copies of R18 (equivalent to 1 - 106 human chondrocytes).

7. NF-kB and AP-1-bearing promoter activity

A 0.7 kb segment at the 5′-flanking region containing NF-kB and AP-1 promoter of the

human matrix metalloproteinase-9 (MMP-9) gene was amplified by PCR using specific primers from the human MMP-9 gene (Accession No. D10051): 5′-ACATTTGCCCGAGCTCCTGAAG (forward/SacI) and 5′-AGGGGCTGCCAGAAGCTTATGGT (reverse/ Hind Ⅲ), as described previously[7]. The pGL2-Basic vector containing a polyadenylation signal upstream from the luciferase gene was used to construct expression vectors by subcloning PCR-amplified DNA of NF-kB and AP-1-bearing promoter into the SacI/HindIII site of the pGL2-Basic vector. The PCR products were confirmed by the their size as determined by electrophoresis and by DNA sequencing. NF-kB and AP-1-bearing promoter plasmid was transfected into human chondrocyte cells using the Lipofectamine reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. To assess the NF-kB and AP-1-bearing promoter luciferase, cells were collected and disrupted by sonication in lysis buffer (25mM tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Firefly luciferase activities were standardized for galactosidase activity.

8. Nuclear extracts and electrophoretic mobility shift assay(EMSA)

Nuclear extract was prepared essentially as described previously[8]. Cultured cells were collected by centrifugation, washed and suspended in a 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 a 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 for 15 min at 4°C. Nuclear extract (1020 μg) was preincubated at 4°C for 30 min with a 100-fold excess of an unlabeled oligonucleotide spanning the -79 MMP-9 cis element of interest. The sequences were as follows: AP-1, CTGACCCCTGAGTCAGCACTT; NF-kB, CAGTGGAATTCCCCAGCC. After this time, the reaction mixture was incubated at 4°C for 20 min in a buffer (25 mM Hepes buffer pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl and 2.5% glycerol) with 2 μg of poly dI/dC and 5 fmol (2×104 cpm) of a Klenow end-labeled (32P-ATP) 30-mer oligonucleotide, which spans the DNA binding site in the MMP-9 promoter. The reaction mixture was electrophoresed at 4°C in a 6% polyacrylamide gel using a TBE (89 mM Tris, 89mM boric acid and 1 mM EDTA) running buffer. The gel was rinsed with water, dried and exposed to X-ray film overnight.

9. Analytical methods

Protein contents were determined by a Protein assay kit of Bio-Rad Laboratories (Richmond, CA, USA).

10. Statistical analysis

All measurements were performed in triplicate and repeated 3 times using age- and sex-matched samples. The results are presented as means±S.D.; n represents the number of experiments. Data were also analyzed using Student's t-test (Sigma Plot software; SPSS, Chicago, IL, USA). P values less than 0.05 were considered significant.($p<0.05$)

Ⅲ. Results

1. Inhibition of IL-1β-induced protein expression by BMK-HAS in human chondrocytes

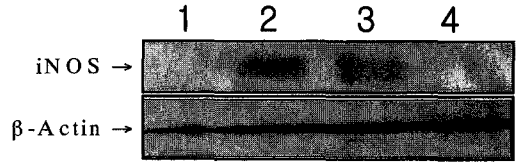

Fig. 1. Inhibition of IL-1β-induced iNOS protein expression by BMK-HAS in human OA chondrocytes
OA chondrocytes were stimulated with IL-1β (2 ng/㎖) and different doses of BMK-HAS for 24 hours, and expression of iNOS protein was determined by Western blotting (a representative Western blot is shown). Data shown are representative of 3 independent experiments. Lane 1, Control; lane 2, IL-1β lane 3, IL-1β+ BMK-HAS (5.0 ㎍/㎖); lane 4, IL-1β+ BMK-HAS (10 ㎍/㎖). Equal loading of protein was verified by probing the same blot for -actin.

To determine whether the suppression of NO production in human chondrocytes was due to the inhibition of iNOS expression, human chondrocytes were treated with different doses of BMK-HAS and IL-1β for 24 hours as described above, and the Western blots were probed with antibodies specific for human iNOS. Our data revealed that the expression of iNOS protein was up-regulated in human chondrocytes stimulated with IL-1β alone and that BMK-HAS inhibited the IL-1β -induced increase in the expression of iNOS protein in human chondrocytes in a dose-dependent manner (Fig. 1). The observed down-regulation of iNOS protein expression was consistent with the nitrite levels in the culture supernatant of chondrocytes that were used to prepare the cell lysate. No effect of BMK-HAS or IL-1β on the protein levels of eNOS or nNOS in human chondrocytes was found (unpublished data).

2. Inhibition of IL-1β-induced activation of NF-kB/p65 transcription factor by BMK-HAS in human chondrocytes

Stimulation by IL-1β leads to the activation of a cascade of adaptor proteins and kinases, resulting in the phosphorylation of IkB by IkB kinases (IKK/). This results in the activation of the transcription factor NF-kB. Since the expression of the iNOS gene is regulated by NF-kB, I determined whether BMK-HAS (10 ㎍/㎖) inhibited the IL-1β-induced activation of NF-kB in human chondrocytes. The DNA binding activity present in nuclear extracts of human chondrocytes showed a rapid increase upon stimulation with IL-1β, reaching a peak between 5 and 30 minutes posttreatment (Fig. 2). However, even at 60 minutes posttreatment, IL-1β-treated chondrocytes had significantly ($P < 0.005$) higher levels of active NF-B than untreated controls. Although the activation of NF-kB was not totally blocked in chondrocytes cotreated with BMK-HAS (10 ㎍/㎖), the levels of DNA-bound NF-kB/p65 were significantly reduced ($P < 0.005$) in comparison with the levels detected in human chondrocytes treated with IL-1β alone (Fig. 2). That there was a reduction in the levels of NF-kB in the nuclei of chondrocytes cotreated with BMK-HAS was also supported by Western blot results that showed reduced levels of NF-B/p65 in the nuclei of these cells in comparison with the nuclei of chondrocytes treated with IL-1β alone

3. BMK-HAS inhibits activation of the NF-kB and AP-1-bearing promoter by IL-1β

I decided to examine the NF-kB and AP-1-bearing promoter to see if BMK-HAS regulated iNOS or COX-2 expression transcriptionally. Chondrocytes were transiently transfected with a plasmid containing a luciferase reporter gene driven by a segment of 710 bp from the NF-kB and AP-1-bearing promoter region (Fig. 3A) and subsequently treated with IL-1β in the absence, or presence of BMK-HAS. IL-1β strongly increased reporter activity, which was attributed to the NF-kB and AP-1-bearing promoter sequence (Fig. 3B). And also, this IL-1β stimulated NF-kB and

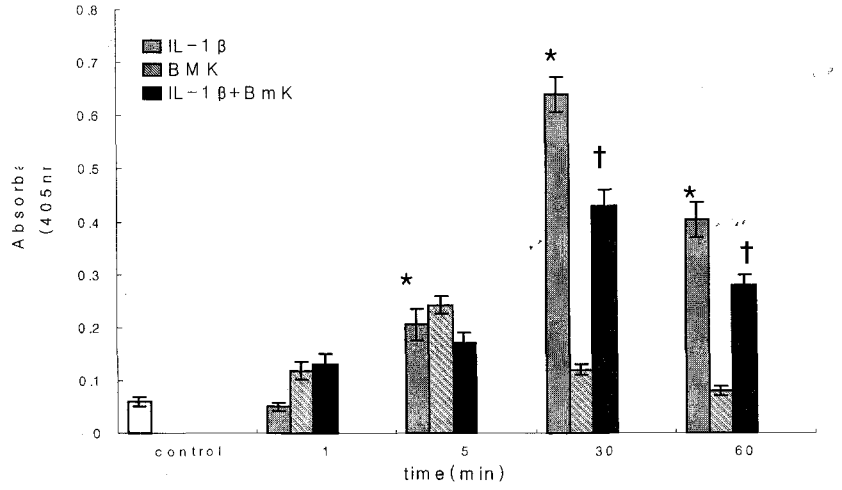

Fig. 2. IL-1β-induced increase in the levels and DNA binding activity of NF-kB/p65 in the nucleus of human OA chondrocytes inhibited by BMK-HAS

OA chondrocytes were stimulated with IL-1β (2 ng/mℓ) and BMK-HAS (10 ㎍/mℓ) for varying times, and activation of NF-kB/p65 was determined by a highly specific ELISA. Specificity of NF-kB binding in this assay was determined by preincubating nuclear protein extracts with a 50-fold excess of kit-supplied wild-type NF-kB or with the mutant NF-kB oligonucleotides.

Values shown are the mean and SD of 3 independent experiments, each performed in duplicate. *, $P < 0.005$ versus control. †, $P < 0.005$ versus IL-1β alone.

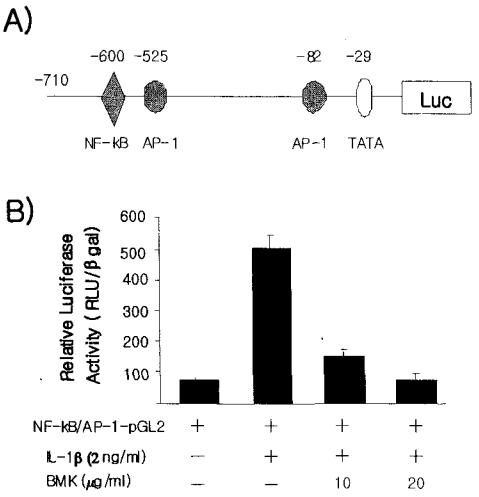

Fig. 3. BMK-HAS inhibition of the IL-1β-induced NF-kB and AP-1-bearing promoter activity

(A) Schematic map of the NF-kB and AP-1-bearing promoter showing the cis-regulatory elements.

(B) Chondrocytes were transiently transfected with NF-kB and AP-1-bearing-pGL2, which contains 710 bp of 5′-promoter of the NF-kB and AP-1-bearing promoter region and then cultured with IL-1β (2 ng/ml) in the presence or absence of indicated concentration of BMK-HAS. Luciferase activity was determined from cell lysates as described under "Experiments".

AP-1-bearing promoter activity was reduced to over 80% following BMK-HAS treatment of chondrocytes, suggesting that the repressive effect of BMK-HAS is due, at least part, to reduced transcription of the NF-kB and AP-1-bearing genes (Fig. 3B).

4. BMK-HAS inhibits the NF-kB and AP-1-bearing promoter by decreasing the NF-kB binding activity

The reporter assay experiments have shown that the response elements for IL-1β stimulation are located within 710 bp upstream of the transcription start site[9]. A distal stimulatory region encompassing an NF-kB element and additional AP-1 binding site has been identified in the inductions of COX-2 and iNOS[10-13]. To examine if IL-1β-induced NF-kB and AP-1-bearing gene expression was associated with an increase in the quantity of NF-kB and AP-1 in the nucleus, EMSA was performed with the

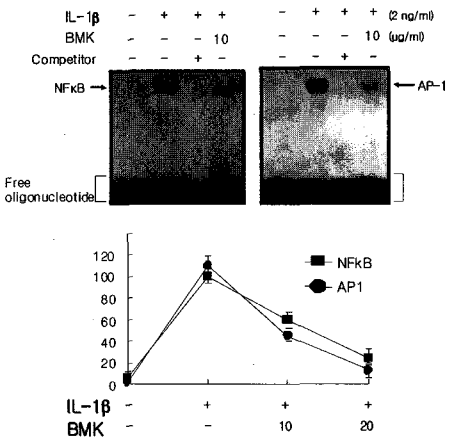

Fig. 4. Effect of BMK-HAS on the IL-1β-induced DNA binding activities of NF-kB and AP-1 motif in chondrocytes

Cells were pretreated with indicated NF-kB and AP-1-bearing promoter BMK-HAS for 40 min in serum free medium were incubated with IL-1β(2 ng/ml) for 24 hour. After incubation, nuclear extracts from the cells were analyzed by EMSA for the activated NF-kB and AP-1 using radiolabeled oligonucleotide probes, respectively.

nuclear extracts of chondrocytes treated with IL-1β(2 ng/ml). In EMSA, nuclear extracts were incubated with a radiolabeled double-stranded oligonucleotide probe with the consensus sequence for NF-kB and AP-1 biding site, respectively and electrophoresed in a 5% nondenaturing polyacrylamide gel. An oligonucleotide derived from the NF-kB and AP-1-bearing promoter sequence spanning this motif was bound specifically with nuclear factors derived from IL-1β-stimulated chondrocytes (Fig. 4A and B). Nuclear extract from chondrocytes treated with IL-1β showed increased binding to the NF-kB and AP-1 motifs (Fig. 4A and B). Next, I examined whether the repressive effect of BMK-HAS on NF-kB and AP-1-bearing gene expression was mediated through these two kinds of motifs. BMK-HAS effectively suppressed the increased NF-kB and AP-1 binding activities (Fig. 4A and B). These data may suggest that BMK-HAS blocks NF-kB and AP-1-bearing gene expression at least in part by decreasing DNA binding of transcription factors NF-kB and AP-1.

These results indicated that BMK-HAS was interfering with an event essential for IL-1β-induced activation and translocation of NF-kB to the nucleus in human chondrocytes. Since activation and translocation of NF-kB to the nucleus are dependent on the phosphorylation of IkB, I next determined the effect of BMK-HAS on the activity of IKK in human chondrocytes using the in vitro kinase assay. My results showed that BMK-HAS had no effect on the IB phosphorylating activity of the IKK complex isolated from human chondrocytes cotreated with BMK-HAS. These results indicated that the observed reduction in the nuclear levels of NF-kB was not due to an inhibition of IKK activity in BMK-HAS-treated human chondrocytes.

Ⅳ. Discussion and conclusion

Natural products of insect origin are still a minor part of traditional medicinal systems in developing countries. There is also a resurgence of interest in insect medicines in western countries as an alternative source of drugs often for intractable diseases such as RA[14]. These issues have shifted the attention toward the development or identification of new compounds that can impinge on the activity of cartilage-degrading factors in an arthritic joint and are better tolerated by the gastrointestinaltract than currently available antirheumatic medicines.

BMK-HAS is widely used in the chronic management and the treatment of RA, particularly, in Korea. However, the mechanism by which the BMK-HAS modify the clinical status of RA are not well understood. Since the clinical treatment with immunosuppressive agents such as cyclosporin A and FK-506 had a beneficial effect in patients with refractory RA[15-16], BMK-HAS might be a useful tool for the treatment of RA. It would be incredible if the drugs as powerful as this did not

have serious toxicity, but further studies will be necessary to answer this question. However, biochemical and metabolic analysis of the constituents of BMK-HAS have to be analyzed in further delineating its mechanisms of action in arthritis.

I have coincided following Oriental remedy claimed to be used in the treatment of rheumatism, bone resorption and related inflammatory diseases. The aim of this study was to investigate the antibone resorption activity of BMK-HAS by using an in vitro screening method based on the inhibitory effects on IL-1β production and gene expression.

Extensive studies using BMK-HAS in many animal model systems have shown that extract is anti-inflammatory and BMK-HAS was inhibitory to the development of type II collagen-induced arthritis in mice, a model of inflammatory polyarthritis[17]. A majority of the biologic effects of BMK-HAS are mediated by one of its principal constituents. Studies have shown that BMK-HAS inhibits the induction and expression of iNOS in mouse macrophages stimulated with LPS. Since high levels of iNOS expression and production of NO are also induced by IL-1β in human chondrocytes, in this study I addressed the question of a possible inhibitory effect of BMK-HAS on IL-1β-induced production of NO in human chondrocytes.

My results showed that BMK-HAS suppressed the IL-1β-induced iNOS mRNA and protein expression and production of NO inhuman osteoarthritis (OA) chondrocytes and that these effects were concomitant with inhibited activation of the transcription factor NF-kB and AP-1. My results thus identify a unique mechanism of action of BMK-HAS for exerting its anti-inflammatory effects in human OA chondrocytes and suggest that BMK-HAS may have beneficial health effects in arthritis. The present study was also undertaken to examine the effects of BMK-HAS on murine macrophage and human neutrophil functions as well as on several enzymes relevant to the inflammatory process.

In other studies, inhibition of iNOS has been shown to protect against both inflammation and cartilage matrix loss in experimental models of arthritis[18-19]. Taken together, these data suggest that agents capable of inhibiting proinflammatory cytokine-induced iNOS activity and NO production may be of potential benefit in both the degenerative and inflammatory joint diseases.

The proinflammatory cytokine interleukin-1β (IL-1β), produced in an arthritic joint by activated synovial cells and infiltrating macrophages, is considered to be one of the most potent catabolic factors in joint diseases[20]. IL-1β induces the production of several mediators of cartilage degradation, such as nitric oxide (NO) and matrix metalloproteinases, and inhibits the concentration of tissue inhibitor of metalloproteinases in arthritic joints[20-21]. IL-1β suppresses the biosynthesis of type II collagen and aggrecan[22] and the proliferation of chondrocytes[23-24], thus inhibiting the repair process in the cartilage.

Cyclooxygenase-2 (COX-2) and iNOS are not generally expressed in resting cells, but are induced following appropriate stimulation with pro-inflammatory agents such as cytokines and Lipopolysaccharide[25]. The activity of these inducible enzymes results in overproduction of PGs and NO, which play a key role in the pathophysiology of arthritis and other inflammatory conditions[26]. NO is also able to enhance the production of tumor necrosis factor-α (TNF-α) and IL-1β, which participate in the macrophage-dependent inflammation[27].

The results indicate that BMK-HAS can control NO and PGE_2 overproduction by selective inhibition of the enhanced expression of both enzymes, thus providing a possible strategy in the treatment of inflammatory diseases. In the present study, I demonstrated that the BMK-HAS inhibited IL-1β-induced production of NO in human chondrocytes. My results further show that the inhibitory effect on NO production was mediated by inhibiting the expression of iNOS mRNA, although I cannot rule out an inhibitory effect of BMK-HAS on the activity of iNOS as

well. The inhibition of NO production by BMK-HAS appears to be a specific effect in suppressing the IL-1β-induced expression of iNOS or production of NO in human chondrocytes. Although BMK-HAS did not completely abolish the production of NO in chondrocytes, low levels of residual NO detected were not toxic to human chondrocytes, since no apoptosis was seen in these cultures.

Several studies have shown that the expression of iNOS is dependent on the activation of the ubiquitously expressed transcription factor NF-kB[28]. My data also show that there are decreased levels of NF-kB/p65 in the nuclei of chondrocytes cotreated with BMK-HAS, suggesting that the inhibitory effect of BMK-HAS on the induction and expression of iNOS is partly a result of reduced levels of activated NF-kB/p65 in the nucleus of chondrocytes. Decreased levels and DNA binding of NF-kB/p65 to its consensus site in human chondrocytes cotreated with BMK-HAS may be of particular significance because this subunit potently transactivates target genes, while the NF-kB/p50 homodimers exert relatively low transactivation of target genes[29]. BMK-HAS repressed IL-1β -induced NF-kB and AP-1-bearing promoter activity by reducing NF-kB and AP-1 binding activity. These findings are the first to show that BMK-HAS inhibits IL-1β -induced inflammation. We also demonstrate for the first time that the BMK-HAS inhibits the NF-kB and AP-1-bearing gene expression on IL-1β-stimulated chondrocytes and this inhibition can be ascribed to the repression of NF-kB and AP-1-bearing promoter activity by decreased NF-kB and AP-1 binding activities. The regulatory elements in the 5′-flanking region of human NF-kB and AP-1-bearing gene have been analyzed previously[10-13]. The IL-1β response element was identified within the 670 bp of the 5′-promoter region of the human gene in tumor cell line[30]. In this study, we first showed that the NF-kB and AP-1-bearing promoter region existing around 710 bp and 23 bp, which contain the NF-kB binding site and AP-1 site, is required for stimulation by IL-1β in chondrocytes. Also, the study here in clearly revealed that the ability of BMK-HAS to reduce NF-kB and AP-1-bearing gene expression in chondrocytes is achieved via reduced NF-kB and AP-1 binding as well as diminished trans-activation of the MMP-9 promoter. BMK-HAS's anti-inflammatory effects seemed to be mediated through transcriptional down-regulation of NF-kB and AP-1-bearing genes. In fact, our studies with the NF-kB and AP-1-bearing promoterrevealed that BMK-HAS regulates this transcription at NF-kB site and AP-1 site. These findings suggest that BMK-HAS may have an anti-inflammatory effect on chondrocytes through the inhibition of NF-kB and AP-1-bearing gene expression, which has been linked to progression of bone resorption and inflammation. Furthermore, we demonstrated that BMK-HAS potently inhibits NF-kB and AP-1 binding activities in repressing IL-1β-induced NF-kB and AP-1-bearing gene expression. The findings of the present study may provide a potential mechanism that explains the anti-bone resorption activity of BMK-HAS. Thus, BMK-HAS may be a therapeutically effective agent, in combination with current treatment modalities, for inhibiting IL-1β-induced cartilage degradation in arthritis.

V. References

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