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원저

Anti-arthritic Effects of Buthus martensi Karsch Herbal Acupuncture, Inhibiting Interleukin-1-induced Expression of Nitric Oxide Synthase and Production of Nitric Oxide in Human Chondrocytes

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국문초록

전갈 약침액의 인체연골세포에서 nitric oxide synthase의 interleukin-1 유도 유전형질 발현과 nitric oxide의 생산의 억제에 관한 연구

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목적 : 면역억제 작용을 지닌 것으로 알려진 전갈약침(BMK)의 IL-1으로 야기된 1차성 골관절염 인체 연골세포에 대한 항염증 효과 및 골 기능 효과에 대해 연구하였다.

방법 : 골관절염 연골에서 채취된 인체 연골세포는 IL-1(2 ng/㎖)에 의해 처리되어졌으며, IL-1과 BMK(10 ㎍/㎖)를 함께 처리한 연골세포와 비교하였다.

결과 : IL-1 단독처리된 연골세포에 비해 BMK가 함께 처리된 연골세포에서 연골세포의 손실과 퇴화의 중요한 요소인 NO의 생산량이 의미있게 저하되었다. IL-1 단독으로 처리된 연골세포보다 IL-1과 BMK가 함께 처리된 연골세포에서 iNOS mRNA의 단백질 합성이 의미있게 감소하였다. 또한, 전사인자로서의 NF-B의 활성화가 IL-1 단독으로 처리된 연골세포에 비하여 BMK가 함께 처리된 군에서 상대적으로 의미있게 억제되었다.

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결론 : 이상의 결과를 종합하면 BMK가 인체 골관절염 연골에 있어서 NF-B 활성화에 의존한 IL-1 유도 염증의 치료상에 효과적인 반응억제제임을 시사하며, 골 세포의 골 재흡수 활동에 효과적임을 시사한다.

Key words : ***Buthus martensi Karsch***, IL-1β, NO, NOS, chondrocytes, OA.

I. Introduction

Among the different scorpion species, *Buthus martensi Karsch*, 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[1]. Nociceptive afferent fibers could be activated to induce excitatory amino acid release from spinal dorsal horn by nociceptive factors *BMK*-I, but the delayed release of GABA was attributed to the modulating role of some antinociceptive components in the venom[2]. The effects of scorpion *Buthus martensi Karsch (BMK)* venom on plasma extravasation and paw withdrawal latency (PWL) to radiant heat was also reported[3]. *BMK* venom by subcutaneous injection under the surface of the rat hindpaw caused dose-dependant increased plasma extravasation. BMK venom may provide a valuable resource for controling a number of inflammatory diseases and identifying potential anti-inflammatory and analgesic drugs. Presently, *Buthus martensi Karsch Herbal-acupuncture (BMK)*, as a traditional Korean immunosuppressive agents and has been widely used in the treatment of some immune-related diseases, especially rheumatoid arthritis (RA) and satisfactory results are obtained[4].

It is known that *BMK* is effective for the treatment of inflammation, gynecological diseases such as osteoporosis and bone resorption, according to the ancient Chinese and Korean medicinal and herbal literature[5]. The *BMK* is a Korean herbal medicines applied in Korea and China as an effective biological response modifier for augmenting host homeostasis of body circulation[5]. The pharmacological action of *BMK* has been limitedly studied in regard to acupuncture diseases. This medicine has been shown to express diverse activities such as immunomodulating, anti-infarction, anti-allergic and anti-inflammatory effects. It is a hemostatic agent, promoting blood coagulation and it is also a cardiac tonic and diuretic ; these actions are probably effected by producing renal vasodilation[6]. Although treatment with *BMK* 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 RA.

Natural products of plant origin are still a major part of traditional medicinal systems in developing countries. There is also a resurgence of interest in herbal medicines in western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis[7]. A literature survey on the *BMK* revealed that there is no scientific evidence of its usefulness in the treatment of RA and osteoporosis. 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.

The proinflammatory cytokine interleukin-1 β (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[8]. 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[8),9]. IL-1 β also suppresses the biosynthesis of type II collagen and aggrecan[10] and the proliferation of chondrocytes[11),12], thus inhibiting the repair process in the cartilage. Additional evidence indicating the involvement of IL-1β in cartilage degradation emerged from studies showing that intraarticular administration of IL-1β into rabbit and mouse joints results in loss of proteoglycans from the cartilage. IL-1 β exerts its inflammatory effects by activating a diverse spectrum of signaling cascades in the cells[13),14] that lead to the induction of inducible nitric oxide synthase(iNOS) and the production of high levels of the second messenger NO in arthritic joints[15].

Constitutive and cytokine-inducible nitric oxide(NO) synthase activities are observed in cultured osteoblast-like cells from various species[16]. Nitric oxide(NO) is involved in various pathophysiological events in many diseases[16] and is produced from L-arginine by nitric oxide synthase(NOS). Nitric oxide(NO) is also an important signaling molecule in bone and is produced by bone cells and organ cultures in response to diverse stimuli such as proinflammatory cytokines[17], mechanical stress[18] and sex hormones[19]. Three isoforms of NOS are studies and reported. Two constitutive isozymes of endothelial cell NOS (ecNOS) and neuronal NOS(nNOS) which produce less amount of NO with several physical/chemical stimuli, while inducible NOS (iNOS) yields larger amount of NO through synthesis of the enzyme in response to proinflammatory cytokines or bacterial lipopolysaccharidesv[16].

Proinflammatory cytokines such as IL-1β and TNF-α regulate NO production in osteoblasts by activating the inducible NOS[20]. Osteoblasts have been reported to produce NO after induction of iNOS gene by these cytokines[13], and NO may inhibit the bone-resorbing activity in adjacent osteoclasts[21], suggesting a cross-talk between osteoblast and

osteoclast via NO. Nevertheless, the role of NO in osteoblasts is still obscure. Although the effects of NO on osteoclast activity have been studied extensively, less is known of the physiological role that NO plays in regulating osteoblast function. Cytokine-induced NO production has been shown to inhibit osteoblast growth[17] and to stimulate osteoblast apoptosis[20]. Evidence of constitutive NOS activity has also been reported in some osteoblast-like cell lines[22]; however, it is still unclear which isoforms of NOS are responsible for this activity and whether constitutive NO production by osteoblasts plays a role in regulating normal chondrocyte function.

Involvement of NO in the pathogenesis of arthritis is evident from studies showing that high levels of nitrite/nitrate are present in serum and in the synovial fluid of arthritis patients, and the messenger RNA(mRNA) and protein of iNOS have been detected in the synovial tissue of osteoarthritis(OA) patients[15),23]. Generation of NO by chondrocytes is recognized as an important factor in cartilage loss and degradation in arthritic joints[24),25]. This is supported by experimentally derived evidence from animal models that demonstrated a significant relationship between NO production and apoptotic chon- drocyte death in cartilage[24]. Of importance to the present studies are the findings that inhibition of iNOS, the enzyme responsible for the production of high levels of NO in vivo, also protected chondrocytes from apoptosis[26]. In mammalian cells, regulation of iNOS expression is predominantly governed by the ubiquitously expressed transcription factor nuclear factor B(NF-B), which is required for the inducible expression of genes associated with inflammatory responses[27].

BMK has a long history of human consumption with no known serious side effects and extensive studies in many animal model systems have shown that extract is anti-inflammatory and that the constituent polyphenols are potent antioxidants[28]. We have previously shown that consumption of *BMK* was inhibitory to the development of type II collagen-induced arthritis in mice, a model of inflammatory polyarthritis[28].

A majority of the biologic effects of *BMK* are mediated by one of its principal constituents. Studies have shown that *BMK* inhibits the induction and expression of iNOS in mouse macrophages stimulated with lipopolysaccharide(LPS). Since high levels of iNOS expression and production of NO are also induced by IL-1 in human chondrocytes, in this study we addressed the question of a possible inhibitory effect of *BMK* on IL-1-induced production of NO in human chondrocytes. Our results showed that *BMK* suppressed the IL-1-induced iNOS mRNA and protein expression and production of NO in human OA chondrocytes and that these effects were concomitant with inhibited activation of the transcription factor NF-B. Our results thus identify a unique mechanism of action of a dietary constituent for exerting its antiinflammatory effects in human OA chondrocytes and suggest that consumption of

BMK may have beneficial health effects in arthritis.

Ⅱ. Materials and Methods

1. Materials

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

The aqueous extracts of *BMK* 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* for application in cell culture were suspended in normal saline at a concentration of 1㎎/㎖.

2. Preparation of articular chondrocytes

Macroscopically normal cartilage samples were obtained from OA patients(7 samples) at the time of total joint replacement. 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[29]. Isolated chondrocytes were plated at a density of $1-10^6$/㎖ in 60-mm tissue culture dishes(Corning, Corning, NY) 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-1 and *BMK*

Human chondrocytes($1-10^6$/㎖) were plated in 6-well plates and serum-starved for 12 hours/overnight. The medium was replaced with fresh medium containing recombinant human IL-1(2 ng/㎖), and chondrocytes were incubated for 24 hours in a tissue culture incubator at 37℃ and 5% CO_2. This concentration of IL-1 was chosen based on pilot experiments performed to determine the lowest concentration of IL-1 that will induce the maximum production of NO by OA chondrocytes in vitro. To study the dose-dependence of the effect of *BMK* on IL-1-

induced iNOS expression, chondrocytes were pretreated for 30 minutes with varying concentrations of *BMK* (1-10㎍/㎖) before the addition of IL-1. Controls consisted of chondrocytes incubated in the culture medium without *BMK* or IL-1.

4. Nitrite determination

NO production was determined by estimating the nitrite concentration in 24-hour culture supernatant using a commercially available kit according to the instructions of the manufacturer(R&D Systems). For each experiment, nitrite concentration was quantified in the samples using a standard curve prepared with known concentrations of nitrite. NO production was also assessed by the measurement of the stable NO metabolite, nitrite, in culture medium using the Greiss reaction as previously described[16].

5. Western blot analysis

After the treatments, chondrocyte lysate was prepared as described previously[29] and used for Western blot analysis. For studies involving NF-B, nuclear and cytosolic fractions were prepared. Protein content of the lysates was determined using a detergentcompatible protein assay kit(Bio-Rad, Hercules, CA), and 25g 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-B/p65, iNOS, endothelial cell nitric oxide synthase (eNOS), or neuronal nitric oxide synthase (nNOS) were either rabbit polyclonal antibodies or mouse monoclonal antibodies(Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were blocked with 5% nonfat dried milk or 2% bovine serum albumin in Tris buffered saline (TBS) containing 0.05% 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) 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.

6. NF-B DNA binding activity assay

Cellular levels of active NF-B/p65 in the nuclei of human chondrocytes treated with IL-1 and *BMK* 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-B oligonucleotides supplied in the kit were used as specificity controls and were added to some

wells prior to the addition of chondrocyte lysate.

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

Poly(A)+ RNA was isolated from FLG 29.1 using oligo(dT) affinity chromatography according to Oligotex kit instructions(Quiagen Korea Co., Seoul, Korea). Total RNA from adipose tissue specimens, obtained from women undergoing elective abdominal surgery who gave written consent, was isolated using a commercial kit(Quiagen Co. or Promega Co.). Total cytoplasmic RNA was prepared from human chondrocytes using a commercially available kit according to the instructions of the manufacturer. We used real-time RT-PCR with internal fluorescent hybridization probes in an ABI Prism 7700 detection system(ABI/Perkin Elmer Biosystems, Foster City, CA), which allows the sensitive and specific quantification of targeted mRNA transcripts. The target-specific RT primer(5-CTCTgg TCAAAC-3), PCR primers(forward 5-AgCgg gATgACTTT CCAAgA-3 ; reverse 5-ATAATggACCCCAgg CAAgATT-3), and the TaqMan Probe for iNOS(6FAM-CCATAAggCCAAAggg ATTTTAA CTTgCAg-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 100ng of total RNA prepared from OA chondrocytes as described above. The RNA was mixed with 10M of RT primer, Moloney murine leukemia virus reverse transcriptase, RNase inhibitor, 0.1M DTT, buffer, and dNTPs using a commercially available kit according to the instructions of the manufacturer(Invitrogen, Carlsbad, CA). The quantitative PCR reactions were set up using 5㎕ of cDNA and 20㎕ 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 $1-10^6$ human chondrocytes).

8. Analytical methods

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

9. Statistical analysis

All measurements were performed in triplicate and repeated 3 times using age- and sex-matched samples. Data were analyzed using Student's t-test(SigmaPlot software ; SPSS, Chicago, IL). P values less than 0.05 were considered significant.

Ⅲ. Results

1. Inhibition of IL-1-induced NO production by *BMK* in human chondrocytes.

Primary chondrocytes($1-10^6$/㎖) were stimulated with IL-1(2ng/㎖) or IL-1+*BMK*(10 ㎍/㎖) for 24 hours, and the concentration of nitrite(as an indicator of NO production) was estimated in culture supernatant. Results demonstrated that upon cotreatment with *BMK*, human chondrocytes produced significantly less NO($P<0.01$) when compared with the values obtained in cultures treated with IL-1 only <Fig. 1>.

To confirm that the NO production in human chondrocytes was due to the activity of iNOS and not of other NOS isoforms, human chondrocytes were treated with IL-1 in the presence of aminoguanidine(AG), a selective iNOS inhibitor, and the accumulation of nitrite in culture supernatant was analyzed. As shown in <Fig. 1>, in the presence of AG(1.0mM), IL-1-induced nitrite formation by human chondrocytes was completely abolished, indicating that eNOS or nNOS isoforms were not activated by IL-1 to any significant degree in human chondrocytes.

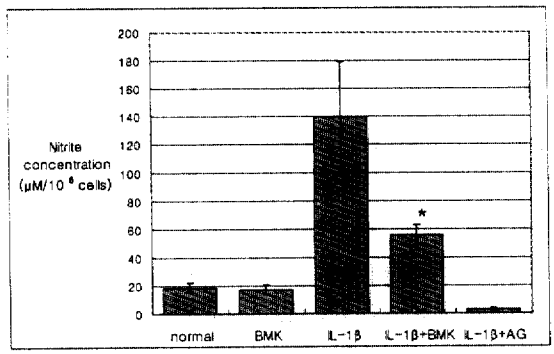

Fig. 1. Inhibition of interleukin-1(IL-1)-induced nitric oxide(NO) production by BMK in human chondrocytes.

Human osteoarthritis chondrocytes were stimulated with IL-1(2 ng/㎖) and BMK(10 ㎍/㎖) for 24 hours. Production of NO was measured in culture supernatant using a commercially available kit. Values shown are the mean and SD of 4 independent experiments, each run in triplicate. *=$P<0.01$ versus treatment.

2. Inhibition of IL-1β-induced iNOS mRNA and protein expression by *BMK* in human chondrocytes

To determine that the inhibition of IL-1β-induced expression of iNOS was due to the inhibition of iNOS induction and not to the inhibition of translation, we analyzed the iNOS mRNA expression in human chondrocytes by real-time quantitative RT-PCR. After 12 and 24 hours of culture, iNOS mRNA expression in IL-1-treated human chondrocytes was severalfold higher in comparison with the levels present in untreated human chondrocytes, which exhibited a constitutive low level of expression <Fig. 2>. Remarkably, and consistent with the above results, *BMK*(100M) was found to inhibit the IL-1-induced increase in the expression level of iNOS mRNA in human chondrocytes at both time points analyzed <Fig. 2>.

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* and IL-1 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 severalfold in human chondrocytes stimulated with IL-1alone and that *BMK* inhibited the IL-1-induced increase in the expression of iNOS protein in human chondrocytes in a dose-dependent manner(data not shown). 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* or IL-1 on the protein levels of eNOS or nNOS in human chondrocytes was found(unpublished data).

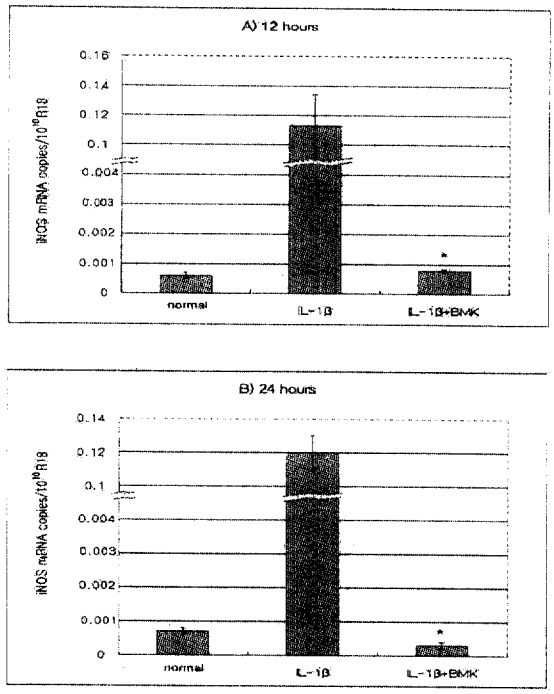

Fig. 2. Inhibition of IL-1-induced inducible nitric oxide synthase(iNOS) mRNA by BMK in human osteoarthritis(OA) chondrocytes.

OA chondrocytes were stimulated with IL-1(2 ng/mℓ) and IL-1 plus BMK(10 μg/mℓ) for 12 and 24 hours, and expression of iNOS mRNA was determined by real-time quantitative reverse transcriptase-polymerase chain reaction. Data shown are representative of 3 independent experiments. See Figure 1 for other definitions.

3. Inhibition of IL-1β-induced activation of NF-B/p65 transcription factor by *BMK* in human chondrocytes

Stimulation by IL-1β leads to the activation of a cascade of adaptor proteins and kinases, resulting in the phosphorylation of IB by IB kinases(IKK). This results in the activation of the transcription factor NF-B. Since the expression of the iNOS gene is regulated by NF-B, we determined whether *BMK* inhibited the IL-1β-induced activation of NF-B 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. 3>. 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-B was not totally blocked in chondrocytes cotreated with *BMK*, the levels of DNA-bound NF-B/p65 were significantly reduced($P<0.005$) in comparison with the levels detected in human chondrocytes treated with IL-1β alone <Fig. 3>. That there was a reduction in the levels of NF-B in the nuclei of chondrocytes cotreated with *BMK* 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-1 alone(results not shown).

Taken together, these results indicated that *BMK* was interfering with an event essential for IL-1-induced activation and translocation of NF-B to the nucleus in human chondrocytes. Since activation and translocation of NF-B to the nucleus are dependent on the phosphorylation of IB, we next determined the effect of *BMK* on the activity of IKK in human chondrocytes using the in vitro kinase assay. Our results showed that *BMK* had no effect on the IB phosphorylating activity of the IKK complex isolated from human chondrocytes cotreated with *BMK* (not shown). These results indicated that the observed reduction in the nuclear levels of NF-B was not due to an inhibition of IKK activity in *BMK*-treated human chondrocytes.

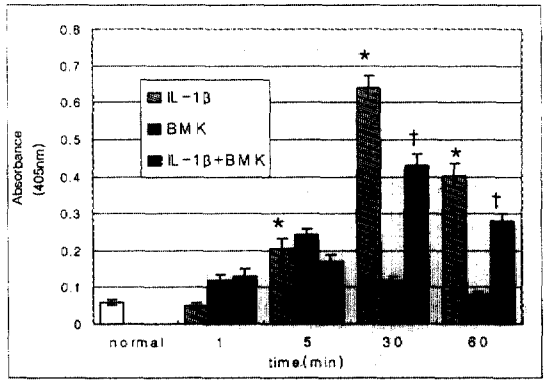

Fig 3. IL-1-induced increase in the levels and DNA binding activity of nuclear factor B(NF-B) /p65 in the nucleus of human osteoarthritis(OA) chondrocytes inhibited by BMK.

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

Ⅳ. Discussion

BMK is widely used in the chronic management and the treatment of RA, particularly, in Korea. However, the mechanism by which the *BMK* modify the clinical status of RA are not well understood. There is general consensus that CD4+ T cells act as initiators of RA, by migrating to the affected joints, recognizing peptides derived from processed antigens, and releasing several types of cytokines[30]. Such cytokines enhance the function of other cells, expecially macrophages to produce pro-inflammatory cytokines including IL-1β and TNF-α[31].

On the other hand, *BMK* treatment suppressed the development of arthritis and immune responses to collagen. Since the clinical treatment with immunosuppressice agents such as cyclosporin A and FK-506 had a beneficial effect in patients with refractory RA[32,33], *BMK* 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* have to be analysed in further delineating its mechanisms of action in arthritis.

A characteristic feature of arthritic joints is the persistence of proinflammatory cytokines produced by the inflamed synovium as well as by chondrocytes in the affected joints[34,35]. It is well known that human chondrocytes are highly responsive to IL-1 and the most striking effect of IL-1 on chondrocytes is to induce the production of NO, which is an important source of highly reactive free oxygen radicals, within the chondrocytes and synoviocytes by inducing the expression of iNOS[15,23]. Although arthritis is present in every population and OA is the most common joint disorder, treatment is still limited to a few classes of drugs, primarily nonsteroidal antiinflammatory drugs and injectable corticosteroids[36~39]. However, while providing relief from pain, none of these agents has been shown to inhibit cartilage breakdown or to inhibit disease progress ; they also have varying degrees of gastrointestinal toxicity[30,37].

Plants used in folk medicine have been aceepted as one of the main sources of drug discovery and development. In Korea, there is a rich treasury of ethnobotanical knowledge and over the past decade we have been widely engaged in the research on this subject. During our field studies, we have coincided following Oriental and herbal remedy claimed to be used in the treatment of rheumatism, bone resorption and related inflammatory diseases : *BMK*. The aim of this study is to investigate the antibone resorption activity of *BMK* by using an in vitro screening method based on the inhibitory effects on IL-1 production and gene expression. Natural products of plant origin are still a major part of traditional medicinal systems in developing countries. There is also a resurgence of interest in herbal me-

dicines in western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis[7]. A literature survey on *BMK* revealed that there is no scientific evidence of its usefulness in the treatment of RA and osteoporosis. Therefore, 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.

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 gastrointestinal tract than currently available antirheumatic medicines. A class of compounds that is gaining attention is the dietary supplements and, recently, glucosamine, a commonly used dietary supplement, has been shown to inhibit cartilage breakdown and disease progress in patients with OA[40]. Other studies have shown that glucosamine prevents the IL-1-induced activation of human chondrocytes through the inhibition of inflammatory processes[37]. Glucosamine is also a potent inhibitor of iNOS induction and production of NO[41], and this may explain its observed cartilage protective effects in OA[40]. In other studies, inhibition of iNOS has been shown to protect against both inflammation and cartilage matrix loss in experimental models of arthritis[26),42]. 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.

In the present study, we demonstrated that the green tea polyphenol *BMK* inhibited IL-1-induced production of NO in human chondrocytes. Our results further show that the inhibitory effect on NO production was mediated by inhibiting the expression of iNOS mRNA, although we cannot rule out an inhibitory effect of *BMK* on the activity of iNOS as well. The inhibition of NO production by *BMK* appears to be a specific effect, because epigallocatechin or sodium gallate alone or in combination was not effective in suppressing the IL-1-induced expression of iNOS or production of NO in human chondrocytes(unpublished data), indicating that the *BMK* structure was important in mediating this effect. Although *BMK* 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(unpublished data).

Several studies have shown that the expression of iNOS is dependent on the activation of the ubiquitously expressed transcription factor NF-B[43]. Our data also show that there are decreased levels of NF-B/p65 in the nuclei of chondrocytes cotreated with *BMK*, suggesting that the inhibitory effect of *BMK* on the induction and expression of iNOS is partly a result of reduced levels of activated NF-B/p65 in the nucleus of chondrocytes. Decreased levels and DNA binding of NF-

B/p65 to its consensus site in human chondrocytes cotreated with *BMK* may be of particular significance because this subunit potently transactivates target genes, while the NF-B/p50 homodimers exert relatively low transactivation of target genes[44].

BMK may be a therapeutically effective agent, in combination with current treatment modalities, for inhibiting IL-1-induced cartilage degradation in arthritis.

V. References

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