Original Article

The Effect of *Aralia Cordata* Thunb and *Cimicifuga Heracleifolia* on Cartilage Protection by the Regulation of Metabolism in Human Osteoarthric Chondrocytes

Shin Ye-ji*, Beak Yong-hyeon*, Park Dong-suk*, Kim Jae-kyu** and Koh Hyung-kyun*

*Dep. of Acupuncture & Moxibustion, College of Oriental Medicine, Kyung Hee University
**Div. of Clinical Medicine, School of Korean Medicine, Pusan National University

국문초록

퇴행성 관절염에 대한 독활·승마 복합처방의 대사조절을 통한 연골보호 효과

신예지* · 백용현* · 박동석* · 김재규** · 고형균*

*경희대학교 한의과대학 침구학교실
**부산대학교 한의학전문대학원 임상의학부

목적 : 퇴행성 관절염은 염증성 사이토카인인 IL-1β에 의해 연골관절이 파괴되고 이로 인해 염증성 사이토카인이 더욱 증가하는 질환이다. 퇴행성 관절염을 치료하기 위해서는 연골 파괴를 가속화시키는 catabolic cytokines의 활성을 줄이고, 성장인자인 anabolic factor의 활성을 증가시는 연골 보호 작용이 있어야 한다. 본 연구에서는 독활·승마 처방(OAH19T)이 catabolic/anabolic 대사 조절에 어떤 영향을 미치는지와 그 신호 전달 기전에 대해 연구하였다. 또한 OAH19T를 구성하는 단미재 및 임상에서 사용되는 COX-2 inhibitor인 Celebrex(CEL)와 효능을 비교 실험하였다.

방법 : 배양된 세포에 IL-1β로 자극한 후 ① glycosaminoglycan(GAG)의 분해 억제 정도, ② OAH19T와 CEL에 대하여 MMP-1과 MMP-3의 유전자 발현 및 활성 억제, ③ Aggrecan 및 Aggrecanases의 유전자 발현 및 활성 억제, ④ OAH19T의 growth factor의 조절 능력, ⑤ MAPK pathway 등을 RT-PCR(reverse transcriptase-polymerase chain reaction), ELISA(Enzyme-linked immunosorbent assay), western blot, viability 측정을 통해 검증했다.

· Acceptance : 2010. 6. 23. · Adjustment : 2010. 8. 4. · Adoption : 2010. 8. 4.
· Corresponding author : Koh Hyung-kyun, Department of Acupuncture and Moxibustion, College of Oriental Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul, Republic of Korea
Tel. 82-2-958-9200 E-mail : koh5795@gmail.com

결과 : 사람 관절 세포에서 ① 독활·승마 각각의 단미재, 임상에서 사용중인 셀레콕시브(CEL), 조인스보다 실험 약물(OAH19T)이 저농도에서 GAG 분해 억제 효과가 우수하였고, 부탄올로 분획한 OAH19B와는 동등한 효과를 보였다. ② OAH19T는 IL-1β에 의하여 활성화된 MMP-1과 MMP-3의 발현을 모두 억제하였으나, CEL은 MMP-1의 발현은 억제하였으나 MMP-3의 발현은 억제하지 못하였다. ③ OAH19T는 IL-1β에 의하여 손상된 Aggrecan을 회복시켰으며 이는 활성화된 Aggrecanase-1과 Aggrecanase-2를 억제시킴으로써 나타난 결과이다. 그러나 CEL의 경우, 손상된 Aggrecan을 회복시키지 못하였다. ④ 배양된 세포는 IL-1β에 의하여 TGF-βII및 TGF-β receptor II의 발현이 억제되었으나, OAH19T는 TGF-βII및 TGF-β receptor II의 발현을 회복시켜 OAH19T가 anabolic한 조절능력이 있음을 시사한다. 그러나 CEL의 경우 growth factor에 대한 조절 능력이 없었다. ⑤ 대사 조절 작용에 대한 기전으로서 MAPK pathway에 대해서 연구한 결과 IL-1β에 의하여 유도된 pERK, pp38 kinase의 활성은 억제하였고, pJNK의 활성은 변하지 않았다. 또한 OAH19T는 연골 세포에 독성이 없었으며 IL-1β에 의해 유도된 세포 증식만을 억제시켰다. 이 결과로, OAH19T가 OA chondrocyte의 탈분화 및 세포 고사를 억제하여 연골보호 및 회복 효과가 있음을 알 수 있었다.

결론 : OAH19T는 이를 구성하는 단미재 및 CEL보다 연골보호 효과가 월등하였고, 이러한 연골보호 효과는 catabolic cytokines/growth factors의 균형으로 대사조절을 통해 연골세포의 탈분화 및 세포 고사를 억제하여 연골보호 및 회복 효과가 있음을 알 수 있었다.

핵심 단어 : 독활, 승마, 골관절염, 관절 보호

Ⅰ. Introduction

Osteoarthritis(OA) is a joint degenerative disease caused by joint overuse, obesity, aging, gender, and cartilage gene mutations[1]. Resorption of cartilage is preceded by an initial excessive synthesis of extracellular matrix and failure of repair processes induced by an imbalance between cytokine-mediated anabolic and catabolic processes[1,2]. Disruption of the balanced network of cytokines/growth factors that normally maintains cartilage metabolism is thought to induce breakdown of extracellular matrix[3].

Interleukin-1β(IL-1β) plays a major role in joint disease, causing overproduction of catabolic activity such as prostaglandin E2, reactive oxygen species (ROS), nitricoxide, and metalloproteinases, which all contribute to the degenerative process[4]. Metalloproteinases of 2 families seem to play major roles in cartilage breakdown. These families are 1) aggrecanases, which play pathological roles at an early stage of disease, especially degrading the proteoglycan which is a major component of cartilage, and 2) matrix metalloproteinases(MMPs) that cleave the aggrecan core protein and are responsible for late stage degradation of both proteoglycan and the collagen network[4,5]. The inhibition of metalloproteinases including aggrecanases and MMPs represent very attractive targets for the development of therapeutics that could alter the progression of OA. The anabolic activity of chondrocytes is sustained by growth factors such as transforming growth factor-β(TGF-β), insulin-like growth factor-1(IGF-I), fibroblast growth factor-2(FGF-2) and bone morphogenetic proteins(BMP). TGF-β signaling starts with binding of TGF-β to TGF-β-RII(Tb-RII), a constitutively active serine/threonine kinase[6,7]. TGF-β has been shown to regulate TGF-β-induced gene expression by activating mitogen-activated protein kinases(MAPKs) such as c-Jun NH2-terminal kinase (JNK) and p38 MAPK pathways as well as the ERK1/2 pathway[8-10]. Several studies indicate that JNK and p38 kinase activation processes are associated with apoptosis, whereas ERK activation is coupled with cell survival. Therefore, coordinated activation and interactions between ERK and p38 MAP kinase allow cells to respond to various genotoxic and survival factors by affecting a

number of downstream targets[11,12].

OAH19T is an extract from the mixture of two oriental herbs, *Aralia cordata* Thunb and *Cimicifuga heracleifolia*, which has been used for treating arthritis in Oriental medicine. *Aralia cordata* has been used to treat arthritis and low back pain. In an *in vitro* study, it has been reported that *Aralia cordata* inhibited COX-2 dependent PGE2 generation and showed effectiveness regarding analgesia, hypothermia, and duration of pentobarbital induced anesthesia[13,14]. In our previous *in vitro* and *in vivo* study, *Aralia cordata* inhibited the cartilage and chondrocyte destruction through the down-regulation of MMP activities and the inhibition of proteoglycan and collagen degradation[15,16]. The rhizoma of *Cimicifuga* species have been widely used as an antipyretic, analgesic, andanti-inflammatory drug in Oriental medicine. In the present study, the author investigated whether if OAH19T has cartilage protective effects and if that is more efficient than the single herb *Aralia cordata* Thunb which has known cartilage protective effects, by comparing OAH19T with other OA drugs in OA chondrocytes.

Ⅱ. Materials and methods

1. Preparation of OAH19T extract and standardization

The *Aralia cordata* Thunb and *Cimicifuga heracleifolia* was purchased from a herbal supplier in Seoul(Korea), and the voucher specimen was deposited at the herbarium of the Pharmacy of Oriental medicine of Kyung Hee Medical Center, Kyung Hee University(Seoul, Korea). The material was authenticated by Professor Kim Nam Jae, Pharmacy of Oriental medicine at Kyung Hee Medical Center, Kyung Hee University. Two hundred grams of *Aralia cordata* Thunb and one hundred gram of *Cimicifuga heracleifolia*(2 : 1) was extracted with 50% (v/v) ethanol-water at 60°C for 8h. The extracted solution was filtered and evaporated in vacuo(OAH-19T, yield 18.7%), and the residue was partitioned between *n*-butanol and water. The *n*-butanol layer was evaporated and lyophilized for a complete removal of the residual solvent to yield dark-brown powder(OAH19B, yield 6.1%). The extract was then filtered with 3MM paper, and ethanol was removed by vacuum rotary evaporation(Eyela, Japan). The concentrate was freeze-dried and its yield was 12.5%. OAH19T was standardized based on the contents of berberine determined using A Waters-600S controller HPLC instrument(Waters Corporation, MA, USA), equipped with a 626 pump, an in-Line degasser AF, a 717 plus auto-sampler and a Waters 996 photodiode array detector(DAD), was used at room temperature. A linear gradient elution of solvent A (ultra-pure water contains 0.1% formic acid, 2mM ammonium acetate) and solvent B(acetonitrile) was applied with the following program. A; 0~20 min, 10~35% B; 20~35min, 35~50% B; 35~45min, 50~85%. Pimaradianoic acid (a) and ferulic acid (b)

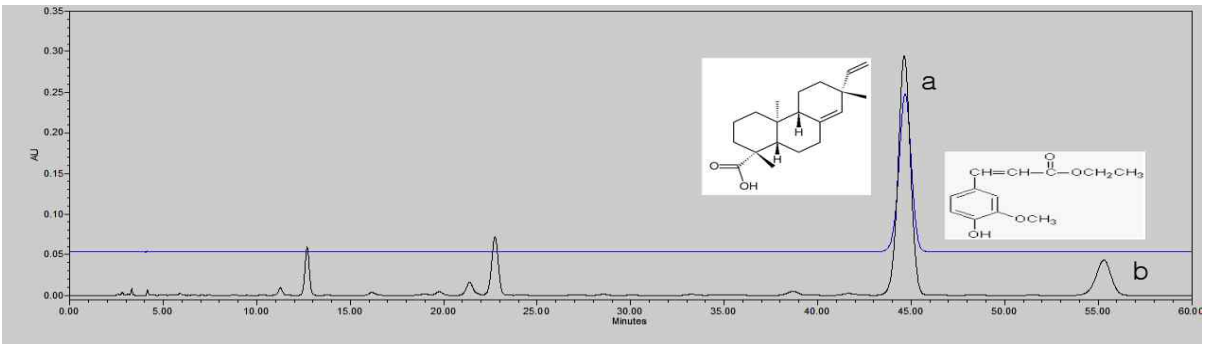

Fig. 1. Representative HPLC chromatogram of pimaradianoic acid, ferulic acid and extracts of OAH19T at the wavelength of 213nm

Pimaradianoic acid (a) and ferulic acid (b) was detected at round 45min and 55min in this system.

was detected at round 45min and 55min in this system(Fig. 1).

2. Preparation of chondrocytes from human osteoarthritic cartilage

Discarded knee cartilage was procured from OA patients(seven samples of hip cartilage) at the time of total joint replacement surgery with the approval of the Institutional Review Board of Hospitals of Kyung Hee University. OA chondrocytes were prepared from macroscopically normal cartilage samples by enzymatic digestion essentially as previously described[17,18], and the viability of the isolated chondrocytes, determined by Trypan blue exclusion assay, was approximately 80%. Isolated chondrocytes were plated at a density of 1 × 106/ml in 60mm tissue culture dishes in Ham's F-12 : DMEM(1 : 1), supplemented with L-glutamine, penicillin-streptomycin, and 10% FBS(completemedium) and allowed to adhere to the tissue culture plates for 72h at 37℃ with 5% CO_2 and 95% air. In some experiments, single passaged chondrocytes were also used.

3. Treatment of chondrocytes with IL-1β and OAH19T

OA chondrocytes(1 × 106/ml) were plated in six well plates in complete medium and incubated for 72h at 37℃ with 5% CO_2 and 95% air. OA chondrocytes were then serum-starved overnight and then the medium was replaced with fresh medium containing recombinant human IL-1b(5ng/ml) or medium with IL-1β + OAH19T or CEL and other samples. OA chondrocytes were incubated for 18h at 37℃ with 5% CO_2 and 95% O_2. Controls consisted of OA chondrocytes incubated without OAH19T or CEL and other samples included in the medium.

4. Glycosaminoglycan(GAG) degradation assay

GAG levels in the culture medium(at 7days from onset of culture) were determined by the amount of polyanionic material reacting with 1, 9-dimethylmethylene blue. 20ml samples were mixed with 100ml DMB reagents(48mg/ml DMB, 40mM glycine, 40mM NaCl, 10mM HCl, pH 3.0) for 30 min at room temperature, and quantified spectro-photometrically in absorbance at 590nm(Spectramax, Molecular Devices, Sunnyvale, CA, USA). All measurements were performed in quadruplicate of three independent experiments using OA chondrocytes. Quantification was performed using a standard curve of chondroitin 6-sulfate from shark cartilage(Sigma) in the range of 0~35mg/ml.

5. Gene expression of reverse transcriptase-polymerase chain reaction(RT-PCR)

Chondrocytes was harvested and lysed with Trizol® reagent(Invitrogen Corporation, CA, USA), and then centrifuged at 12,000rpm for 10min at 4°C. Reverse transcription of 1mg of total RNA was carried out for 60min at 42°C and then 15min at 72°C, using the system for RT-PCR(TaKaRa Biotechnology, Seoul, Korea), which contained RT buffer, oligo(dT) 12-mer, 10mM dNTP, 0.1M dithiothreitol, reverse transcriptase, and RNase inhibitor. PCR using specific primers for each cDNA was carried out in a PCR reaction volume of 20mL(as supplied by TaKaRa, Korea), supplemented with 2.5units of TaKaRa TaqTM 1.5mM each dNTP, 1 × PCR buffer, and 20 pmol of each primer. Amplification reactions were performed by using MMP-1, MMP-3, aggrecan, aggrecanase-1 and aggrecanase-2 primers. The oligonucleotide used for PCR studies are described in Fig. 1. An equal volume from each PCR was analyzed by 1.5% agarose gel electrophoresis, and ethidium bromide-stained PCR products were evaluated. Marker gene expression was normalized to GAPDH expression in each sample.

Signal intensity was quantified with the Gel Doc EQ(BIO-RAD Laboratories, Milan, Italy).

6. Enzyme-linked immunosorbent assay

The levels of MMP-1, MMP-3, TGF-β, and TGF-βRII were measured by human enzyme-linked immunosorbent assay(ELISA) kits(R&D System Inc, MN, USA) respectively, according to the manufacturer's instructions. The conditioned media were collected on 21days, centrifuged at 1,500rpm for 5min in order to remove cell debris, and then stored at -70°C until use. For each sample, MMP-1, MMP -3, TGF-β, and TGF-βRII level were measured.

Conditioned medium from onset of chondrocytes culture was incubated(in the presence of 1% w/v BSA in PBS/Tween 20(0.05% v/v)) for 2h at 25°C on a 96-well plate(Biosource) containing a monoclonal antibody that recognizes KS chains and according to the manufacturer, is not impacted by other non-KS glycosaminoglycans, including hyaluronic acid, chondroitin sulfate, and heparin sulfate. Fragments containing ARGSVIL neoepitope were detected using biotinylated mAb OA-1(PIERCE, IL, USA). Levels of bound biotinylated mAb OA-1 were detected using 1μg/ml streptavidin-HRP and TMB as substrate. Absorbance readings following acidification were determined in a microplate reader at a wavelength of 450nm. Calibration curves for standard ARGSVIL peptide were run in parallel, and the amounts of ARGSVIL peptide produced in hydrolytic reactions were calculated from the calibration curves.

7. Western blot analysis

OA chondrocyte lysate was prepared on ice in a lysis buffer(Invitrogen, Carlsbad, CA, USA). The proteins(10μg/lane) were size-fractionated by 10% SDS-polyacylamide gel(Invitrogen) electrophoresis under reducing conditions, and transferred onto Hybond-C nitrocellulose membranes(Amersham Biosciences, NJ, USA). For the detection of phosphorylation of ERK, JNK and p38-MAPK and non-phosphorylation state-specific, polyclonal antibodies (Cell Signaling Technologies, MA, USA) were used. Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.05% Tween-20(TBST). Primary antibodies were diluted in TBST and incubated with the blots for 12h at 4℃, washed with fresh TBST and incubated with a 1 : 3,000 dilution of horseradish peroxidase(HRP) conjugated anti-mouse or anti-rabbit IgG(Sigma-Aldrich Co, MO, USA) in blocking buffer for 1h at room temperature. After washing, the protein bands were visualized with the ECL kit(Amersham Biosciences, NJ, USA) and Kodak Bio-Max film.

8. Measurement of cell viability

Cell viability measurement was performed in 96-well plates. Chondrocytes were plated at 2 × 104 cells/well in 96-well plate containing 100μl of medium. After 18h, the cells were treated with different concentrations(0~1,000μg/ml) of test samples in the presence or absence of IL-1β and TGF-βRII in a volume of 100μl. After 72h incubation at 37℃, 10μl of BrdU were added to each well, and the samples were incubated for a further 6h at 37℃. Cells were fixed, anti-BrdU-POD was added, and then detection was performed by the TMB substrate reaction. The reaction product was quantified by ELISA reader at 480~650mm.

9. Statistical analysis

Results are expressed as means±S.D. Statistical significance was determined using one way analysis of variance(ANOVA) followed by Dunnett's post-hoc test. p-values less than 0.05 were considered statistically significant.

Ⅲ. Results

1. Effect of OAH19T on GAG degradation of IL-1β stimulated human osteoarthritic chondrocytes

An initial experiment was performed to demonstrate the inhibition of GAG degradation in human osteoarthritic chondrocytes in order to compare the efficacy of cartilage protection of *Aralia cordata* Thunb, *Cimicifuga heracleifolia*, andmixture of *Aralia cordata* Thunb and *Cimicifuga heracleifolia*(OAH19T). *Aralia cordata* Thunb, *Cimicifuga heracleifolia*, and OAH19T in doses ranging from 40 to 200μg/ml showed dose-dependent inhibition of GAG degradation.

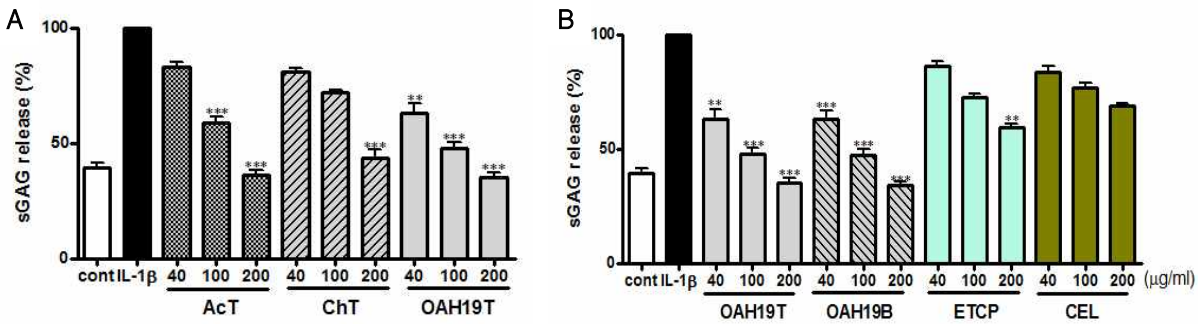

Fig. 2. Effect of OAH19T on GAG degradation in IL-1β stimulated human osteoarthritic chondrocytes

A : GAG degradation of Aralia cordata Thunb, *Cimicifugaheracleifolia*, andOAH19T is shown as a percentage of the cumulative release of chondrocytes.

B : Comparative effect of OAH19T with OAH19B or joins(ETCP) or celecoxib(CEL) on IL-1β-stimulated GAG degradation of chondrocytes. Values are mean±SEM. ** : $p<0.01$ and *** : $p<0.001$ compared with IL-1β.

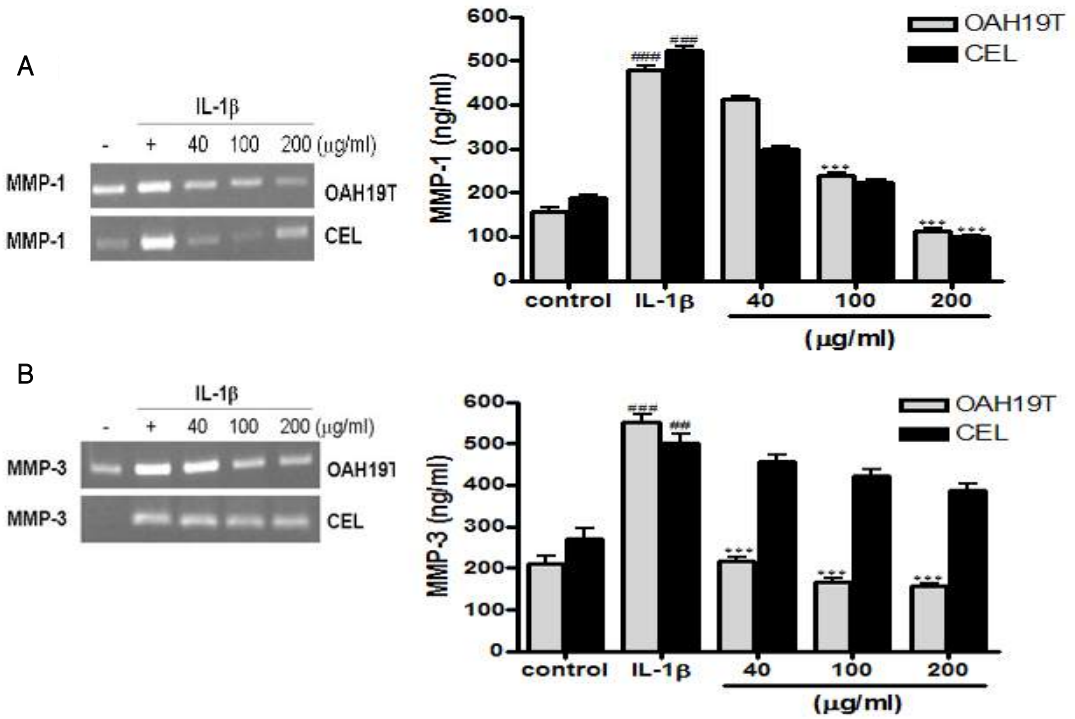

Fig. 3. Effects of OAH19T on the level of matrix proteinases of IL-1β stimulated human osteoarthritic chondrocytes

A : The level of MMP-1 was determined by RT-PCR and ELISA assay.

B : The level of MMP-3 was determined by RT-PCR and ELISA assay. These experiments were repeated twice with similar results. ## : $p<0.01$, ### : $p<0.001$ compared with control, *** : $p<0.001$ comparedwithIL-1β.

Aralia cordata Thunb, *Cimicifuga heracleifolia*, and OAH19T showed inhibition of GAG degradation in dose-dependent manners, and OAH19T showed superior effect compared to single herbs(Fig. 2A). Another experiment was performed to compare the efficacy of cartilage protection of OAH19T with a butanol fraction(OAH19B), Joins(ETCP) or Celebrex (CEL) on IL-1β-stimulated GAG degradation of chondrocytes. ETCP and CEL showed mild dose-dependent inhibition, whereas OAH19T and OAH19B showed marked inhibition on IL-1β-stimulated GAG degradation of chondrocytes(Fig. 2B). The results of OAH19T and OAH19B were almost the same(Fig. 2B).

2. Effects of OAH19T on the level of matrix proteinases in IL-1β stimulated human osteoarthritic chondrocytes

In order to demonstrate the cartilage protective effect on matrix proteinases, OAH19T and CEL in doses ranging from 40 to 200μg/ml were applied on IL-1β stimulated human osteoarthritic chondrocytes, and then RT-PCR and ELISA were performed. The results of RT-PCR showed that osteoarthritic chondrocytes stimulated with IL-1β alone had increased the expression of MMP-1 compared to control, while the expression of MMP-1 decreased when co-treated with OAH19T or CEL. The results of ELISA showed that osteoarthritic chondrocytes stimulated with IL-1β alone had increased levels of MMP-1 compared to control, while the levels of MMP-1 were decre-

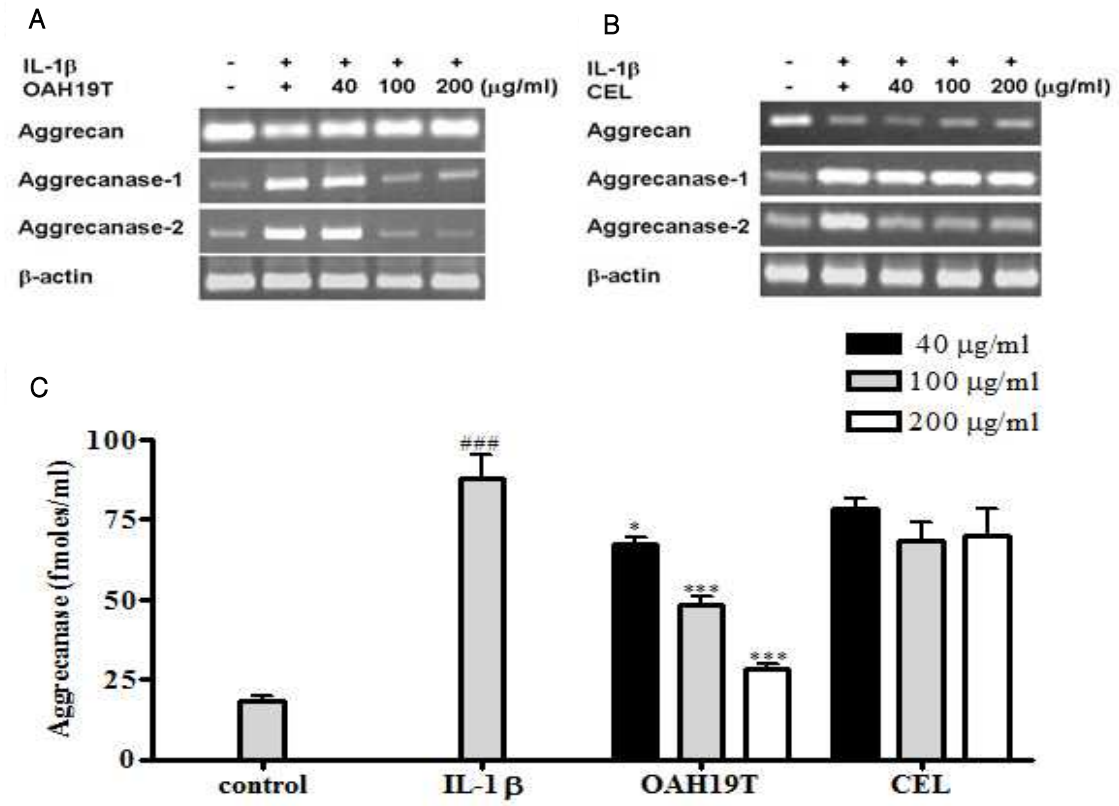

Fig. 4. Effect of OAH19T on the level of aggrecan and aggrecanases of IL-1β stimulated human osteo- arthritic chondrocytes

A and B : The gene expression of aggrecan, aggre- canase-1 and aggrecanase-2 was determined by RT-PCR.
C : The level of aggrecanase was estimated by OA-1 sandwich ELISA. Conditioned medium from chondrocyte culture was digested with hyaluronidase, chondroitinase ABC, and evaluated in the OA-1 mAb sandwich ELISA.
: $p<0.001$ compared with control, * : $p<0.05$ and *** : $p<0.001$ compared with IL-1β.

ased when co-treated with OAH19T or CEL in dose-dependent manners(Fig. 3A). The levels of MMP-3 also significantly increased when stimulated with IL-1β, but the levels were markedly decreased when co-treated with OAH19T nearly under the level of controls in dose-dependent manners. On the contrary, there were no significant differences between control and IL-1β and CEL co-treatment groups (Fig. 3B). The inhibition of MMP-1 by OAH19T was similar to that by CEL, but inhibition of MMP-3 by OAH19T was superior to that by CEL.

3. Effect of OAH19T on the levels of aggrecan and aggrecanases in IL-1β stimulated human osteoarthritic chondrocytes

In order to demonstrate that the cartilage protective effect by inhibiting aggrecan breakdown through inhibiting activity of aggrecanases, we performed RT-PCR to determine the gene expression of aggrecan, aggrecanase-1 and aggrecanase-2, and ELISA to estimate the levels of aggrecanases. The results of RT-PCR showed that osteoarthritic chondrocytes stimulated with IL-1β alone had increased the intensity of the bands of aggrecanase-1 and 2 compared to controls, while the both bands became dim when co-treated with OAH19T(Fig. 4A). On the contrary, when co-treated with CEL, only the expression of aggrecanase-2 slightly decreased, and the expression of aggrecanase-1 was not inhibited on IL-1β stimulated human osteoarthritic chondrocytes(Fig. 4B). The result of ELISA showed that the levels of ggrecanases were almost undetectable in the culture medium, while significant increases were seen on aggrecanases activity in cartilage explants culture with IL-1β-induced inflammation. OAH19T in doses from 40 to 200μg/ml showed

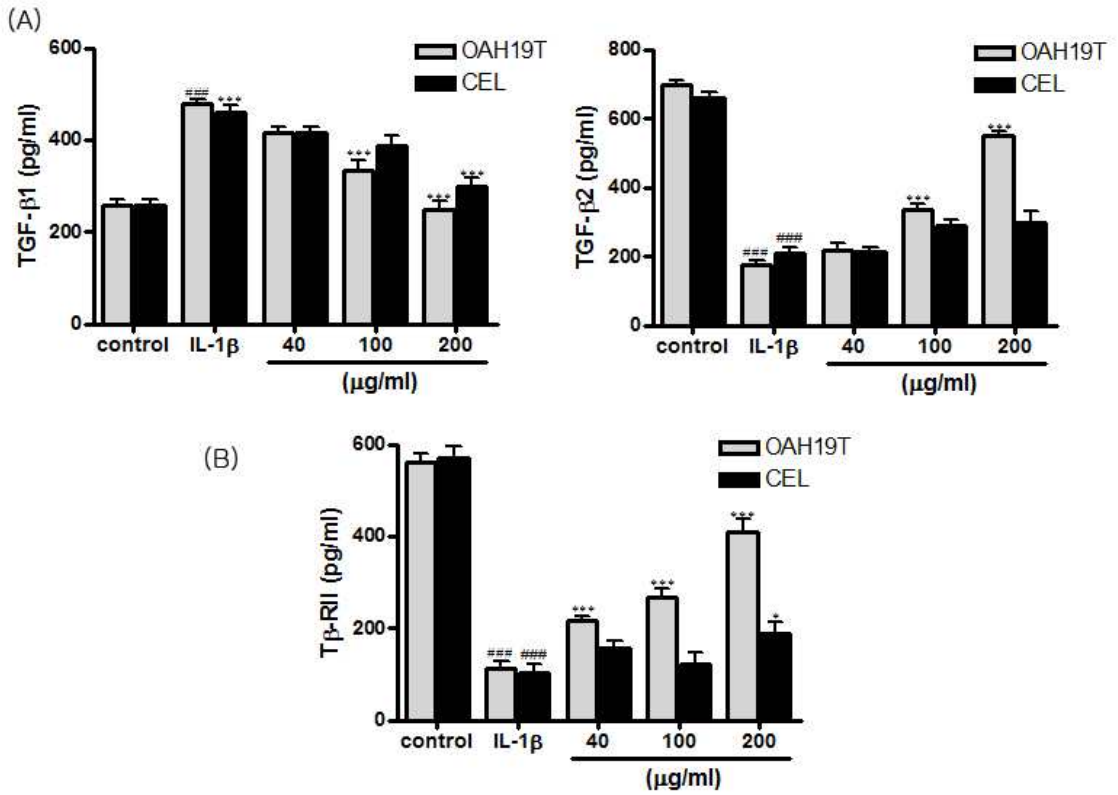

Fig. 5. Effect of OAH19T on the levels of TGF-β and TGF-β receptor II in IL-1β-stimulated human osteoarthritic chondrocytes

The levels of TGF-β1, TGF-β2 and TGF-β receptor II were estimated by ELISA assay.
: $p<0.001$ compared with control, * : $p<0.05$ and *** : $p<0.001$ compared with IL-1β.

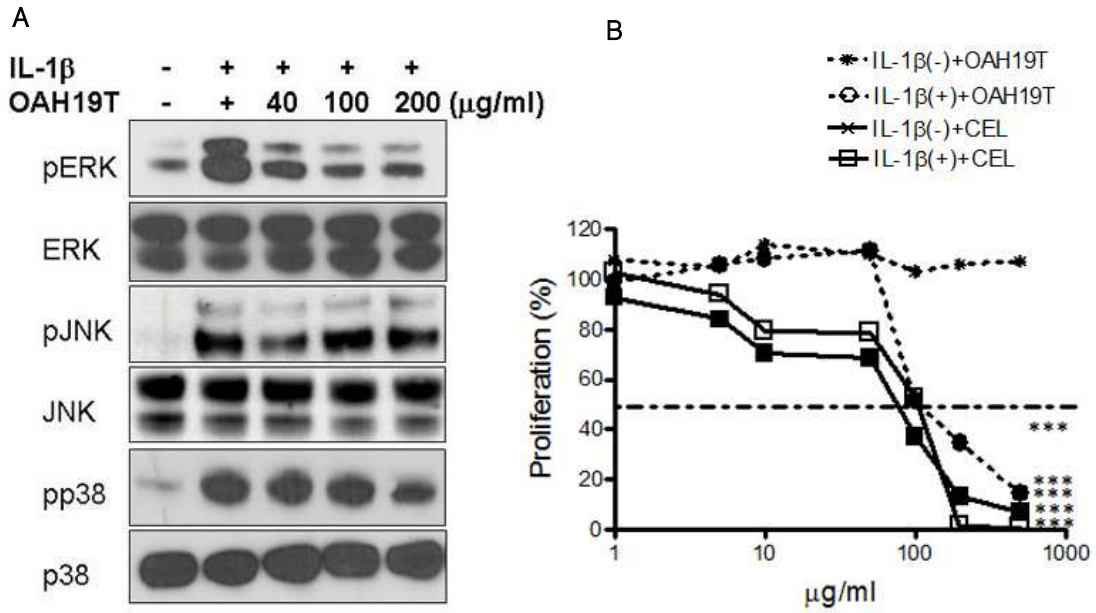

Fig. 6. OAH19T inhibits IL-1β stimulated chondrocytes degradation through MAPK pathways in human osteoarthritic chondrocytes

A : Effect of OAH19Tbyco-treatmentwithIL-1β-receptor inhibitor (IL-1βR) on the expression of pERK, pJNK and pp38 MAPK proteins in IL-1β-stimulated chondrocytes.

B : Effect of OAH19T on the viability of IL-1β-stimulated human osteoarthritic chondrocytes.

*** : $p<0.001$; IL-1β (+) treated OAH19T group compared with IL-1β (-) treated OAH19T group. [a]$p<0.001$: TGF-βR+IL-1β treated OAH19T group compared with IL-1β (+) treated OAH19T group. [b]$p<0.001$: TGF-β R+IL-1β treated CEL group compared with IL-1β (+) treated CEL group.

marked reduction of activity of aggrecanases in a dose-dependent manner, while CEL rarely inhibited aggrecanases activity(Fig. 4C). Notably, the reduction of aggrecanases by OAH19T was superior to the effect by CEL(Fig. 4C).

4. Effect of OAH19T on the level of TGF-β and TGF-β receptor II in IL-1β-stimulated human osteoarthritic chondrocytes

TGF-β play a crucial role in cartilage homeostasis, and therefore it was necessary to determine the effects of OAH19T on the expression of TGF-β and its receptor II. The results of ELISA showed that osteoarthritic chondrocytes stimulated with IL-1β alone had increased the levels of TGF-β1 compared to controls, while the levels of TGF-β1 were significantly decreased when co-treated with OAH19T in a dose-dependent manner(Fig. 5A). The levels of TGF-β2 and TGF-β receptor II(Tb-RII) were markedly decreased in the presence of IL-1β, but were increased in a dose-dependent manner with OAH19T in doses ranging from 40 to 200μg/ml the levels of TGF-β and Tb-RII, while CEL rarely influenced the levels(Fig. 5).

5. OAH19T inhibits IL-1β stimulated chondrocyte degradation through MAPK pathways in human osteoarthritic chondrocytes

To determine whether the preceding effects could be associated with modulation of downstream pathways following IL-1β receptor occupation, the author analyzed the activation state of MAP kinases(ERK, JNK, p38 kinase). The result showed that ERK, JNK and p38-MAPK were phosphorylated in osteoarthritic chondrocytes stimulated with IL-1β in vitro, phosphorylation of ERK and p38-MAPK were

markedly inhibited in the presence of OAH19T, and JNK was rarely inhibited(Fig. 6A).

The author next examined the effect of OAH19T on the viability of human osteoarthritic chondrocytes in order to determine its cytotoxicity. OAH19T alone has not shown cytotoxicity even in high doses, whereas CEL alone has shown cytotoxicity of human osteoarthritic chondrocyte. OAH19T co-treated with IL-1β inhibited the proliferation of human osteoarthritic chondrocytes compared to the control group (Fig. 6B). These results present that OAH19T rarely has cytotoxicity even in high doses and inhibits the cytotxicity in IL-1β-stimulated human osteoarthritic chondrocyte(Fig. 6B).

Ⅳ. Discussions

In the present study, the author evaluated the effects of OAH19T on cartilage protection in human osteoarthritis chondrocytes. The present study shows that the crude extract of OAH19T can modulate the metabolism and pathological dysfunction of extracellular matrix, and that the efficacy of OAH19T was superior to that of a COX-2 inhibitor, Celebrex.

OAH19T, a new herbal agent, is a mixture of 2 Oriental medicinal herbs. A problem in using natural herbal material is the difficulty in standardization of efficacy, which is partly due to factors such as differences in region of origin, harvest period, and time cultivated. Therefore, the author analyzed the major components in order to set a standard for use of OAH19T in practice and in medicine development(Fig. 1).

Aggrecanase-1 and -2 have been identified as the known enzymes that are most efficiently capable of cleaving aggrecan[19,20] by modulating cartilage turnover in articular cartilage[19,20]. In mice, aggrecanase-2(butnotaggrecanase-1) is responsible for disease progression in a surgically-induced model of OA[20,21]. However, questions remain regarding the relative contribution of aggrecanase-1 and aggrecanase-2 in human disease[20-22]. Matrix metalloproteinases(MMPs) are a large group of enzymes that play a crucial role in tissue remodeling as well as in the destruction of cartilage and bone in an arthritic joint due to their ability to degrade a wide variety of extracellular matrix components[23-26]. Among the various MMPs, MMP-1 is mainly synthesized by chondrocytes or fibroblasts in connective tissues and is the most abundant member of the MMP-family, which plays an important role in the breakage of cartilage collagen[24]. MMP-3 is capable of cleaving the aggrecan core protein as well as type II collagen in cartilage[25]. Studies have documented that in arthritic joints, degradation of type II collagen is excessive due to increased cleavage by MMPs[27,28].

Matrix proteinases cause irreversible cartilage damage[22,29]. Therefore, modulating the catabolic factors and anabolic factors would be reasonable therapeutic targets for the treatment of osteoarthritis. Especially, aggrecanase-1 is commonly detected in the joint fluids in rheumatoid arthritis and osteoarthritis and expressed by the chondrocytes in diseased cartilages, suggesting that aggrecanase-1 plays an important role in the cartilage destruction in such joint diseases[30]. Previousstudies suggested that MMP-1 and MMP-3 are responsible for the release of proteoglycan and collagen[31,32].

OAH19T markedly prevents GAG release, which is associated with the down-regulation of catabolic factors including aggrecanase-1, aggrecanase-2, MMP-1 and MMP-3 in IL-1β-treated human osteoarthritis chondrocytes. It also modulates the anabolic growth factors, increases TGF-β2 and Tb-RII level, and decreases TGF-β1 without cytotoxicity in IL-1β-treated human osteoarthritis chondrocytes. Surprisingly, OAH19T has a more marked effective on cartilage protection than single herbs or existing medicines(ETCP and CEL) on IL-1β-stimulated GAG degradation of chondrocytes.

Also, the erosion of cartilage in OA is associated with a progressive alteration of the TGF-β system, including reduced expression of TGF-β isoforms and receptors(mainly Tb-RII)[33,34]. This process could be caused by enhanced IL-1 expression in OA,

since one *in vitro* study showed that the cytokine exerts inhibitory effects on the TGF-β system[34]. TGF-β is a multifunctional cytokine involved in crucial biological processes such as development, extracellular matrix synthesis, cell proliferation/differentiation, and tissue repair[33]. TGF-β exerts opposite effects on cartilage protection from interleukin-1ß on cultured rabbit articular chondrocytes[6]. Injection of TGF-β in naive murine knee joints results in an increase in proteoglycan synthesis and proteoglycan content of articular cartilage[35]. Moreover, intra-articular injection of TGF-β during experimental arthritis resulted in protection from proteoglycan loss[36]. In addition, effectsof IL-1, such as inhibition of cartilage proteoglycan synthesis and release of cartilage proteoglycan content, could be blocked by local application of TGF-β[37,38]. This demonstrates that TGF-β is able to counteract the deleterious effects of IL-1, a cytokine considered as a key mediator during erosive joint diseases.

The present results show for the first time that OAH19T can prevent the reduction of TGF-β2 expression and particularly that of Tb-RII. Together, these data suggest that OAH19T could contribute to the anabolic activity of chondrocytes and maintain their homeostasis, and may also be of potential benefit in the treatment of both degenerative and inflammatory joint diseases.

MAPKs are the family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, including stress[38,40]. MAPKs are serine/threonine kinases that, upon stimulation, phosphorylate their specific substrates at serine and/or threonine residues. Such phosphorylation events can either positively or negatively regulate substrate, and thus the entire signaling cascade activity. Thus, the MAPK signaling pathways modulate gene expression, mitosis, proliferation, motility, metabolism, and programmed death 'apoptosis'[11,39-44].

The author searched for the signaling pathways that could be responsible for the effects of OAH19T on gene expression. Exposing chondrocytes to the pro-inflammatory cytokine IL-1β in monolayer culture led to phenotypical dedifferentiation, finally resulting in apoptosis cell death. Indeed a set of catabolic cytokines, including IL-1β and TNF-a, are known to play an essential role in the pathogenesis of OA[45]. The results of the present study suggest that OAH19T inhibited the gene expression of MMPs and aggrecanases by suppressing the phosphorylation of extracellular signal-regulated kinases(Erk1/Erk2) and p38kinase, without significantly altering the phophorylation of JNK. These kinases are known to regulate MMP expression[46].

For this reason, inhibiting the modulation of MAPK may be an important target for selective inhibition of the IL-1β-induced catabolic events in OA chondrocytes. Recently, several investigators also have reported on the beneficial effects of various growth factors such as FGF-2, IGF-1 and TGF-β on chondrogenic differentiation[47-49]. The results of the present study strongly support that OAH19T shows a cartilage protective effect which works by modulating catabolic cytokine/growth factor through the regulation of MAPK pathway.

Although inhibited phophorylation of ERK was observed in IL-1β stimulated chondrocytes treated with OAH19T, this did not have any effect on the viability in IL-1β non-stimulated OA chondrocytes. This suggests that OAH19T exerts its chondroprotective effects by inhibiting the dedifferentiation of OA chondrocytes. The present results also showed that OAH19T had an inhibitory effect on the IL-1β stimulated phosphorylation of p38 MARK. This indicates that p38 MAPK may be associated with chondroprotective effects by inhibiting the apoptosis of OA chondrocytes. Taken together, this suggests that the chondroprotective effect of OAH19T is mediated by inhibiting pERK and p38 MAPK cascade.

In summary, the results demonstrate that OAH19T may be an effective therapeutic agent for inhibiting IL-1β-induced cartilage degradation in joint diseases by modulating the expression of cartilage-specific matrix proteins, TGF-β, and Tb-RII. And the effect against cartilage destruction is more potent than that of the specific COX-2 inhibitor in OA chondrocytes.

Ⅴ. Conclusions

The author performed experiments on osteoarthritic chondrocyte separated from OA patients using OAH19T, and the results are as follows.

1. Suppression of glycosaminoglycan(GAG) degradation was measured in cultured osteoarthritic chondrocytes after IL-β stimulation. In low doses, OAH19T showed significantly superior suppression compared to *Aralia cordata* Thunb, *Cimicifuga heracleifolia*, CEL or Joins, and similar suppression compared to butanol-fractionated OAH19B.
2. OAH19T suppressed IL-1β activation of both MMP-1 and 3, while CEL suppressed only MMP-1.
3. OAH19T induced IL-1β mediated aggrecan damage recovery through suppression of aggrecanase 1 and 2. CEL did not cause aggrecan recovery.
4. IL-1β suppressed TGF-βII and TGF-β receptor II expression, and OAH19T reversed this suppressive effect, showing that it has anabolic modulating effects. CEL did not show such effects on growth factor.
5. OAH19T suppressed IL-1β mediated pERK and pp38 kinase activation, while not suppressing pJNK activation. OAH19T did not have cytotoxicity on chodrocytes and only suppressed IL-1β induced cell proliferation.

These results show that OAH19T produces its chondroprotective and cartilage healing effects by suppressing OA chondrocyte dedifferentiation and apoptosis.

Ⅵ. References

1. Poole AR. An introduction to the pathophysiology of osteoarthritis. Front Biosci. 1999 ; 4 : D662 - 70.
2. Aigner T and Dudhia J. Phenotypic modulation of chondrocytes as a potential therapeutic target in osteoarthritis : a hypothesis. Ann Rheum Dis. 1997 ; 56 : 287 - 91.
3. Dinarello CA and Moldawer LL. Proinflammatory and Anti inflammatory Cytokines in Rheumatoid Arthritis. Thousand Oaks, CA : A Primer for Clinicians Amgen Inc 1999.
4. Lark MW, Bayne EK, Flanagan J, Harper CF, Hoerner LA, Hutchinson NI et al. Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. J Clin Invest. 1997 ; 100 : 93 - 106.
5. Westling J, Fosang AJ, Last K, Thompson VP, Tomkinson KN, Hebert T et al. ADAMTS4 cleaves at the aggrecanase site(Glu373-Ala374) and secondarily at the matrix metalloproteinase site (Asn341-Phe342) in the aggrecan interglobular domain. J Biol Chem. 2002 ; 277 : 16059-66.
6. Pujol JP. TGF-β and osteoarthritis : In vivo veritas? Osteoarthritis and cartilage. 1997 ; 7 : 439-40.
7. Wrana JL, Attisano L, Wieser R, Ventura F and Massaguè J. Mechanism of activation of the TGF-β receptor. Nature. 1994 ; 370 : 341.
8. Sano Y, Harada J, Tashiro S, Gotoh-Mandeville R, Maekawa T and Ishii S. ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-beta signaling. J Biol Chem. 1999 ; 274 : 8949-57.
9. Shirakabe K, Yamaguchi K, Shibuya H, Irie K, Matsuda S, Moriguchi T, Gotoh Y, Matsumoto K and Nishida E. TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. J Biol Chem. 1997 ; 272 : 8141-4.
10. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, and Matsumoto K. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 1995 ; 270 : 2008-11.

11. Chang L and Karin M. Mammalian MAP kinase signalling cascades. Nature 2001 ; 410 : 37-40.
12. Robinson MJ, Cobb MH. Mitogenactivated protein kinase pathways. Curr Opin Cel Biol. 1997 ; 9 : 180-6.
13. Chowdhury TT, Salter DM, Bader DL, Lee DA. Signal transduction pathways involving p38 MAPK, JNK, NFkappaB and AP-1 influe- nces the response of chondrocytes cultured in agarose constructs to IL-1βeta and dynamic compression. Inflamm Res. 2008 ; 57 : 306-13.
14. Dang NH, Zhang X, Zheng M, Son KH, Chang HW, Kim HP et al. Inhibitory constituents against cyclooxygenases from Aralia cordata Thunb. Arch Pharm Res. 2005 ; 28 : 28-33.
15. Baek YH, Huh JE, Lee JD, Choi DY, Park DS. Effect of Aralia cordata extracts on cartilage protection and apoptosis inhibition. Bioll Pharm Bull. 2006 ; 29 : 1423-30.
16. Park DS, Huh JE, Baek YH. Therapeutic effect of Aralia cordata extracts on cartilage protection in collagenase-induced inflammatory arthritis rabbit model. J Ethnopharmacol. 2009 ; 125 : 207-17.
17. Ahmed S, Wang N, Lalonde M, Goldberg VM, Haqqi TM. Green tea polyphenol epigallocatechin-3-gallate(EGCG) differentially inhibits interleukin-1β-induced expression of matrix metalloproteinase-1 and -13 in human chondrocytes. J Pharmacol Exp Ther. 2004 ; 308 : 767-73.
18. Rasheed Z, Anbazhagan AN, Akhtar N, Ramamurthy S, Voss FR, Haqqi TM. Green tea polyphenol epigallocatechin-3-gallate inhibits advanced glycation end product-induced expression of tumor necrosis factor-alpha and matrix metalloproteinase-13 in human chondrocytes. Arthritis Res Ther. 2009 ; 11 : R71.
19. Westling J, Fosang AJ, Last K, Thompson VP, Tomkinson KN, Hebert T et al. ADAMTS4 cleaves at the aggrecanase site(Glu373-Ala374) and secondarily at the matrix metalloproteinase site (Asn341-Phe342) in the aggrecan interglobular domain. J Biol Chem. 2002 ; 277 : 16059-66.
20. Tortorella MD, Malfait AM, Deccico C, Arner E. The role of ADAM-TS4(aggrecanase-1) and ADAM-TS5(aggrecanase-2) in a model of cartilage degradation. Osteoarthritis Cartilage. 2001 ; 9 : 539-52.
21. Glasson SS, Askew R, Sheppard B, Carito BA, Blanchet T, Ma HL et al. Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. Arthritis Rheum. 2004 ; 50 : 2547-58.
22. Powell AJ, Little CB, Hughes CE. Low molecular weight isoforms of the aggrecanases are responsible for the cytokine-induced proteolysis of aggrecan in a porcine chondrocyte culture system. Arthritis Rheum. 2007 ; 56 : 3010-19.
23. Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JF. Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. J ClinI nvest. 1989 ; 84 : 678-85.
24. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases : role in arthritis. Front Biosci. 2006 ; 11 : 529-43.
25. Lin PM, Christoper Chen CT, Torzilli PA. Increased stromyelin-1(MMP-3), proteoglycan degradation (3B3- and 7D4) and collagen demage in cyclically load injuried articular cartilage. Osteoarthritis and Cartilage. 2004 ; 12 : 485-96
26. Dumond H, Presle N, Pottie P, Pacquelet S, Terlain B, Netter P et al. Site specific change in gene expression and cartilage metabolism during early experimental osteoarthritis. Osteoarthritis Cartilage. 2004 ; 12 : 284-95.
27. Kim JH, Ryu KH, Jung KW, Han CK, Kwak WJ, Cho YB. SKI306X suppresses cartilage destruction and inhibits the production of matrix metalloproteinase in rabbit joint cartilage explants culture. J Pharmacol Sci. 2005 ; 98 : 298-306.
28. Dahlberg L, Billinghurst RC, Manner P, Nelson F, Webb G, Ionescu M et al. Selective enhancement of collagenase-mediated cleavage of resident type II collagen in cultured osteoarthritic cartilage and arrest with a synthetic inhibitor that spares collagenase 1(matrix metalloprotei-

nase 1). Arthritis Rheum. 2000 ; 43 : 673-82.
29. Fosang AJ, Last K, Maciewicz RA. Aggrecan is degraded by matrix metalloproteinases in human arthritis. Evidence that matrix metalloproteinase and aggrecanase activities can be independent. J Clin Invest. 1996 ; 98 : 2292-9.
30. Nagase H, Kashiwagi M. Aggrecanases and cartilage matrix degradation. Arthritis Res Ther. 2003 ; 5 : 94-103.
31. Dodge GR, Jimenez SA. Glucosamin sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis and Cartilage. 2003 ; 11 : 424-32.
32. Shingleton WD, Ellis AJ, Rowan AD, Cawston TE. Retinoic acid combines with interleukin-1 to promote the degradation of collagen from bovine nasal cartilage: Matrix Metalloproteinase-1 and -13 are involved in cartilage breakdown. J Cellular Biochem. 2000 ; 79 : 519-31.
33. Redini F, Mauviel A, Pronost S, Loyau G, Pujol JP. Transforming growth factor ß exerts opposite effects from interleukin-1ß on cultured rabbit articular chondrocytes through reduction of interleukin-1 receptor expression. Arthritis Rheum. 1993 ; 36 : 44-50
34. Boumediene K, Conrozier T, Mathieu P, Richard M, Marcelli C, Vignon E, Pujol JP. Decrease of cartilage transforming growth factorbeta receptor II expression in the rabbit experimental osteoarthritis potential role in cartilage breakdown. Osteoarthritis Cartilage. 1998 ; 6 : 146-9.
35. van Beuningen HM, van der Kraan PM, Arntz OJ and van den Berg WB. Transforming growth factor-b stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. Lab Invest. 1994 ; 71 : 279.
36. Glansbeek HL, van Beuningen HM, Vitters EL, van der Kraan PM, and van den Berg WB. Stimulation of articular cartilage repair in established arthritis by local administration of transforming growth factor-β into murine knee joints. Lab Invest. 1998 ; 78 : 133.
37. van Beuningen HM, van der Kraan PM, Arntz OJ and van den Berg WB. In vivo protection against interleukin-1-induced articular cartilage damage by transforming growth factor- β1 : age-related differences. Ann Rheum Dis. 1994 ; 53 : 593.
38. van Beuningen HM, van der Kraan PM, Arntz OJ and van den Berg WB. Protection from interleukin 1 induced destruction of articular cartilage by transforming growth factor : studies in anatomically intact cartilage in vitro and in vivo. Ann Rheum Dis. 1993 ; 52 : 185.
39. Seger R and Krebs EG. The MAPK signaling cascade. FASEBJ. 1995 ; 9 : 726-35.
40. Waskiewicz AJ and Cooper JA. Evolutionary conservation of Xenopus laevis mitogenactivated protein kinase activation and function. Curr Opin Cell Biol. 1995 ; 7 : 798-805.
41. Tibbles LA and Woodgett JR. The stressactivated protein kinase pathways. Cell Mol Life Sci. 1999 ; 55 : 1230-54.
42. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell. 2000 ; 103 : 239-52.
43. Johnson GL and Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. 2002 ; 298 : 1911-2.
44. Werlen G, Hausmann B, Naeher D and Palmer E. Signaling life and death in the thymus : timing is everything. Science. 2003 ; 299 : 1859-63.
45. Fernandes JC, Martel-Pelletier J and Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. Biorheology. 2002 ; 39 : 237-46.
46. Liacini A, Sylvester J, Li WQ, Zafarullah M. Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1(AP-1) and nuclear factor kappa B(NF-kappa B) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. Matrix Biol. 2002 ; 21 : 251-62
47. Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M and Martin I. Specific growth factors during the expansion and redifferentiation of adult human articular chondro-

cytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem. 2001 ; 81 : 368-77.

48. Mandl EW, Jahr H, Koevoet JL, van Leeuwen JP, Weinans H, Verhaar JA and van Osch GJ. Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture. Matrix Biol. 2004 ; 23 : 231-41.
49. Darling EM and Athanasiou KA. Growth factor impact on articular cartilage subpopulations. Cell Tissue Res. 2005 ; 322 : 463-73.