Full Paper

SKI306X Suppresses Cartilage Destruction and Inhibits the Production of Matrix Metalloproteinase in Rabbit Joint Cartilage Explant CultureJoo-Hyon Kim¹, Keun-Ho Ryu¹, Ki-Won Jung¹, Chang-Kyun Han¹, Wie-Jong Kwak¹, and Yong-Baik Cho^{1,*}¹Life Science Research Center, SK Chemicals, 600 Jungja-1-Dong, Changan-Ku, Suwon-Si, Kyungki-Do 440-745, South Korea

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Abstract. SKI306X was previously found to have cartilage protective effects in the experimental osteoarthritis (OA) model. To investigate the chondro-protective benefits of SKI306X for its capacity in altering changes in cartilage metabolism and molecular mechanisms of cartilage protective action, SKI306X is studied in rabbit cartilage explants culture. To investigate the protective effect of SKI306X on cartilage catabolism, we assessed collagen degradation in rabbit cartilage explants treated with interleukin-1 α up to 3 weeks. To examine the reaction mechanism, matrix metalloproteinase (MMPs) were investigated by fluorimetric and Western blotting analysis. In addition, its effects on the activation process of proenzyme MMP-3 were determined by gelatin zymography. SKI306X significantly inhibited collagen degradation and inhibited the activities of several MMPs. Total MMPs activities in cultured medium were substantially increased in the third week at the time of collagen degradation with the absence of SKI306. However, the introduction of SKI306X decreased MMPs activities in cultured medium. Furthermore, Western blotting analysis proved that these inhibitory effects of this drug were the result of inhibiting MMPs expression. SKI306X also inhibited the activation of proenzyme MMP-3 to the active form of MMP-3. These results indicate that SKI306X inhibits matrix degradation by down regulating MMPs expression and secretion, inhibition of MMPs activity, and inhibiting activation of MMP-3 during the collagen breakdown process.

Keywords: SKI306X, osteoarthritis, hydroxyproline, matrix metalloproteinase

Introduction

Osteoarthritis (OA) and rheumatoid arthritis (RA) are degenerative joint diseases characterized by progressive loss of articular cartilage, subchondral bone remodeling, spur formation, and synovial inflammation, in particular, degradation of collagen and proteoglycan (PG). The integrity of these macromolecules is vital to cartilage and joint function (1). OA is believed to be a consequence of mechanical and biochemical events that result in an imbalance between the synthesis and degradation of articular cartilage matrix (2–4). Chondrocytes regulate cartilage metabolism under both normal and pathological conditions. These cells initiate the rapid degradation of PG from cartilage in response to inflammatory cytokines, such as interleukin-1 α (IL-1 α) and tumor necrosis factor α (TNF- α), but this component of

matrix is quickly replaced. However, Collagen is much less readily released from tissue and when degradation occurs, the structural integrity of the tissue is irreversibly lost (2, 3, 5, 6).

SKI306X, an extract purified from a mixture of three oriental herbs, *Clematis mandshurica*, *Trichosanthes kirilowii*, and *Prunella vulgaris*, is known to serve multiple functions such as anti-inflammation, immunomodulation, and the activation of blood microcirculation (7, 8). In previous studies, we reported the protective effects of SKI306X on rabbit articular cartilage and compared it with other OA drugs using in vivo models (9). A double blind and placebo-controlled study also confirmed the clinical efficacy and good tolerability of SKI306X in patients with OA in the knee (10). In another clinical study, this herbal extract was well tolerated by patients and demonstrated to have clinical efficacy comparable to that of diclofenac SR (slow released) (11). Another clinical study is underway to

*Corresponding author. FAX: +82-31-240-8492
E-mail: ybcho@skchemicals.com

determine the efficacy of SKI306X in RA patients. However, its chemistry of cartilage protective action still remained to be determined.

The chondrocytes of articular cartilage synthesize a number of proteinases that are capable of degrading the component molecules of this specialized extracellular matrix. The use of class-specific proteinase inhibitors indicates that major activities responsible for catabolism of PG and collagen are attributable to zinc-dependent metalloproteinase. There are circumstantial *in vitro* and *in vivo* evidences indicating a significant role of matrix metalloproteinase (MMPs) in cartilage destruction in arthritic diseases including OA. MMPs are involved in pathological processes such as metastatic cancer and arthritic diseases and are key enzymes in OA and RA. MMPs are synthesized and secreted as proenzymes in response to stimulants including IL-1 α and TNF- α (2, 3, 5, 6). MMPs are an enzyme super family of at least 21 members, which can be classified into subgroups of collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10, -11), gelatinases (MMP-2, -9), and membrane-type 1 (MMP-14). MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-8 (collagenase-2), and MMP-13 (collagenase-3) have all been suggested to be active in the degradation of cartilage in OA. MMP-3 is capable of cleaving aggrecan core protein, as well as type II collagen (in the amino-terminal telopeptide), *in vitro*, but it is not clear if it is involved in degradation of these proteins in cartilage (12, 13).

These observations suggest that inhibition of MMPs synthesis or activity may be an important factor in OA pathology. To examine the mechanism of cartilage protective effects of SKI306X, we investigated its inhibitory effects on the MMPs production (induced by IL-1 α) on MMPs activities upon their production and in activation process of proenzyme MMP-3 to active MMP-3 in rabbit cartilage explants.

Materials and Methods

Preparation and composition of SKI306X and its herbal components

SKI306X was prepared by extracting a mixture of three medical herbs [dried roots of *Clematis mandshurica*, dried roots of *Trichosanthes kirilowii*, and dried flowers and stems of *Prunella vulgaris* at 1:2:1 (w/w) ratio, respectively] with 30% (v/v) ethanol-water. After the extracted solution was filtered and evaporated *in vacuo*, the residue was partitioned between *n*-butanol and water. The *n*-butanol layer was evaporated *in vacuo* and lyophilized for a complete removal of the residual solvent to yield dark-brown powder. SKI306X was

standardized for quality control, according to a previous report (7). SKI306X was analyzed to find standard materials by HPLC. We identified oleanolic acid and rutin in SKI306X as standard materials (7). *Clematis mandshurica*, *Trichosanthes kirilowii*, and *Prunella vulgaris* were also prepared by the above method.

Cartilage explant cultures

Articular cartilages were obtained from hock joints of individually housed (MJ-161C; Myung Jin Instrument Co., Daejeon, Korea) 5-week-old rabbits (Damool Science, Taejeon, Korea). The articular cartilage explants were obtained by following the methods described by Saito et al. (13), Sandy et al. (14), Yamada et al. (12). Briefly after articular surfaces were surgically exposed under sterile condition, approximately 200–220 mg of articular surfaces per joint were removed and submerged into complete medium (DMEM, supplemented with heat-inactivated 5% FBS and 100 U/ml of penicillin-streptomycin; Gibco BRL, Carlsbad, CA, USA). They were then rinsed several times with the complete medium and incubated for 1 to 2 days at 37°C in a 5% humidified CO₂/95% air incubator for stabilization. The complete medium was replaced with a basal medium (DMEM, supplemented with heat-inactivated 1% FBS, 10 mM HEPES, and 100 U/ml of penicillin-streptomycin). Approximately 50 to 60 mg of cartilage pieces were placed in 24-well plates and treated with specified concentrations of test agents. After 1 h of pretreatment, 5 ng/ml of IL-1 α (R&D Systems, Minneapolis, MN, USA) was added to the culture medium and incubated further at 37°C in a 5% humidified CO₂/95% air incubator. The culture medium was collected several times at various intervals and stored at –20°C until assay initiation of glycosaminoglycan (GAG) and hydroxyproline (OHPro). The 15-day-cultured media treated by test materials were assayed for collagen degradation inhibition effect. Dexamethasone and diclofenac sodium were purchased from Sigma (St. Louis, MO, USA). Rofecoxib was synthesized at Life Science Research Center of SK Chemicals (Suwon-si, Korea).

GAGs measurements

The amount of sulphated GAGs in the medium at the end of reaction reflecting the amount of PG degradation was determined by the 1,9-dimethyl-methylene blue method using a commercially available kit (The Blyscan proteoglycan & glycosaminoglycan assay; Newtownabbey, Northern Ireland) according to the instructions of the manufacturer (15).

Collagen degradation inhibition

OHPro release was assayed (as a measure of collagen degradation) by a modified Bergman and Loxley microtiter plate method (16). Chloramine T (7% (w/v)) was diluted 1:4 in acetate-citrate buffer (57 g sodium acetate, 37.5 g tri-sodium citrate, 5.5 g citrate acid, 385 ml propan-2-ol/1 water). *p*-Dimethylaminobenzaldehyde (DAB: 20 g in 30 ml 60% perchloric acid) was diluted 1:3 in propan-2-ol. Specimens were hydrolyzed in 6 M HCl for 20 h at 105°C, and the hydrolysate was neutralized by drying over NaOH *in vacuo*. The residue was dissolved in water and 40 μ l of the sample or a standard (OHPro: 5–30 μ g/ml) was added to a microtiter plate together with chloramine-T reagent (25 μ l) and DAB reagent (150 μ l) was added after 4 min. The plate was covered and heated to 60°C for 35 min, cooled down to room temperature, and the absorbance at 560 nm was read.

Cytotoxicity assay

Lactate dehydrogenase (LDH) assays were performed on day 7 and 14 media to assess the viability of explanted tissue using the LDH assay kit (IVD Lab Co., Ltd., Suwon, Korea).

Western blotting analysis

MMPs in the culture medium were analyzed by Western blotting. Equal volume of medium samples were boiled for 5 min with 2.5% mercaptoethanol and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 10–20% gradient gels under reducing conditions. After electrophoresis of protein onto a polyvinylidene fluoride membrane (GVHP Durapore filter; Milipore Corp., Bedford, MA, USA), the membrane was blocked with 5% bovine serum albumin and probed with mouse anti-rabbit MMP-3 or mouse anti-human MMP-13 antibody (Calbiochem, Seoul, Korea) at a dilution of 1:500 for 1 h. A second antibody reaction was carried out for 1 h using horse radish peroxidase conjugated anti-mouse IgG antibody from sheep (Amersham Pharmacia Biotech Korea, Seoul, Korea) at a dilution of 1:1000, followed by a reaction with ECL Western blotting detection reagent (Amersham Pharmacia Biotech Korea). The bands of interest were detected by an X-ray film (Sigma).

Fluorimetric analysis of MMPs activities

Rabbit joint cartilage explants were cultured as described above. The (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitro-phenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH₂ (M1895; Bachem, Torrance, CA, USA) was used to evaluate the total MMPs activities in conditioned media. This substrate has been

efficiently cleaved by all MMPs tested so far. The (3-[2,4-dinitro-phenyl]-L-2,3-diaminopropionyl)-Pro-Tyr-Ala-Tyr-Trp-met-Arg-OH (Bachem) was used to evaluate the purified human recombinant proenzyme MMP-3 (prostromelysin; Oncogene, Darmstadt, Germany) activity and (7-methoxycoumarin-4-yl)acetyl-Pro-Cha-Gly-Nva-His-Ala-(3-[2,4-dinitro-phenyl]-L-2,3-diaminopropionyl)-NH₂ (Bachem) was used to evaluate the purified human recombinant proenzyme MMP-13 (procollagenase 3, Calbiochem) activity. The activation of MMPs was achieved by reaction with *p*-aminophenylmercuric acetate (APMA) at 37°C in 0.1 M Tris-HCl, pH 7.5 for 3 h. These assays were performed at 37°C using an Amico-Bowman Luminiscence spectrophotometer Series 2 linked to a computer running the FLU-SYS software (Thermoelectron Corporation, San Jose, CA, USA). The substrate and media were diluted in 0.1 M Tris-HCl, pH 7.5, containing 0.1 M CaCl₂ and 0.05% (v/v) Triton X-100. The steady state cleavage rate was recorded and standardized as the amount of product produced in 1 min per cartilage explants.

Gelatin zymography analysis of proenzyme MMP-3 activation inhibition

Interaction between proenzyme MMP-3, SKI306X, and *Trichosanthes kirilowii* were analyzed by gelatin zymography (17). Purified recombinant human proenzyme MMP-3 can be activated by APMA *in vitro*. SKI306X and *Trichosanthes kirilowii* were pre-incubated for 10 min and incubated for the activation process of proenzyme MMP-3 with 50 ng proenzyme MMP-3 at 37°C in 50 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 0.05% Triton X-100 for 3 h by 1 mM APMA. Samples were mixed with 10 μ l of 4 $\times$ sample buffer (0.5 M Tris-HCl, pH 6.8, containing 40% glycerol, 8% sodium dodecyl sulfate) (SDS; Bio-rad, Hercules, CA, USA) and 0.004% bromophenol blue (Bio-rad) at room temperature for 30 min to allow SDS coating on the protein. Electrophoresis was performed on 10% SDS-polyacrylamide gels containing 0.5 mg/ml gelatin. Then these gels were washed twice for 15 min with 0.25% Triton X-100. To visualize proteinases, gels were incubated for 16 h at room temperature in incubation buffer containing 50 mM Tris-HCl, pH 7.2, 10 mM CaCl₂, 10% (v/v) Triton X-100, and 0.02% sodium azide. Gels were stained with 0.1% Coomassie blue (R-250, Bio-Rad) in 10% acetic acid and 40% methanol solution. Gelatinolytic activities by enzymes were determined by examining clear zones in stained gels, which indicate the lysis of gelatin.

Statistics

Values are expressed as the mean and standard deviation.

tion (S.D.) of quadruplicate assay. Statistical significances among groups were tested using Sigma Stat (Jandel Co., San Rafael, CA, USA) by one-way analysis of variance (ANOVA) and Dunnett's test. Differences were considered significant when P was less than 0.05. All experiments were performed at the minimum of three times, each time with three or more independent observations.

Results

SKI306X effects on collagen degradation in cartilage explant culture

Previously we reported the protective effects of SKI306X on PG degradation (9). SKI306X interfered with the rhIL-1 α -mediated degradation of PG in a concentration-dependent manner in rabbit cartilage explant culture. In this study, we tested the effect of SKI306X on collagen degradation and its protective effect against collagen degradation. In LDH assays, SKI306X and *Trichosanthes kirilowii* did not change the levels in culture medium up to several weeks (Fig. 2B). Rabbit cartilage explants were cultured in the presence of IL-1 α to determine whether SKI306X affects collagen degradation. Explants were cultured for 21 days with once a week medium change, and the collagen released into medium was measured as % release of OHPro. The release of OHPro was negligible in control cartilage explants medium (no IL-1 α present), whereas the medium containing IL-1 α induced a dramatic release of OHPro. No detectable levels of collagen degradation were observed in the culture medium treated with IL-1 α until day 10. After day 17, OHPro was significantly released (Fig. 1). However, the release of OHPro was considerably reduced when SKI306X was introduced to the medium at the concentration of 0.1 to 0.3 mg/ml. *Trichosanthes kirilowii*, components of SKI306X, also effectively inhibited collagen degradation at 0.3 and 0.1 mg/ml. *Prunella vulgaris* had a weak inhibitory effect against collagen degradation. On the other hand, the other components of SKI306X, *Clematis mandshurica*, did not show any significant inhibitory effects on collagen degradation (data not shown). Rofecoxib did not inhibit collagen degradation when tested up to 10 μ M. Dexamethasone did not show any inhibitory effect against collagen degradation at low concentration and showed an effect only at high concentration (Fig. 2A). SKI306X has multifunctional effects: anti-inflammation, analgesic, immuno-modulation, activation of blood microcirculation (7), and cartilage protection. SKI306X and its component herbals showed different effects on these targets. Especially *Trichosanthes kirilowii* showed an efficient effect of cartilage

Fig. 1. Time-course on release of GAG and OHPro fragments from rabbit cartilage in the presence of IL-1 α . Rabbit cartilage explants were stimulated with IL-1 α (5 ng/ml). At various times up to day 21, the culture supernatants were removed and the percentages of total collagen released into the media were determined by GAG and OHPro assay. Values are expressed as the mean $\pm$ S.D. of quadruplicate assays.

protection (Fig. 2). Other components herbs had other effects (7). Therefore, SKI306X can be estimated to have effect on OA patients clinically. In this study, we investigated the mechanism of collagen degradation inhibition by SKI306X.

Western blotting of culture medium

To study the mechanism of the inhibition of IL-1 α -induced collagen degradation by SKI306X, the semi-quantitative analysis of MMP-3 and MMP-13 expression in the culture supernatants were performed using Western blotting. Western blot analysis showed that on day 15, SKI306X and *Trichosanthes kirilowii* significantly down regulated MMP-3, MMP-13 expressions. The cartilage treated with IL-1 α released MMP-3 and MMP-13. When SKI306X and *Trichosanthes kirilowii* were added to the explants culture, release of these MMPs into explants medium was significantly inhibited at concentrations from 0.03 to 0.3 mg/ml. Dexamethasone also significantly inhibited the release of these MMPs at a concentration of 10 μ M (Fig. 3). These results suggest that the inhibition of the expression of these MMPs might be concerned with the prevention of cartilage degradation by SKI306X.

SKI306X effects on the expression of MMPs in cartilage explant culture

We showed MMP-3 and -13 expressed in these

Fig. 2. Effects of SKI306X and *Trichosanthes kirilowii* on collagen degradation. A: Inhibitory effects of SKI306X and *Trichosanthes kirilowii* on OHPro release in rabbit cartilage in the presence of IL-1 α . Rabbit cartilage explants were cultured in medium containing various concentrations of herbal extracts and control agents in the presence of IL-1 α (5 ng/ml). On day 15, the culture supernatants were collected, and the percentages of the total collagen released were determined by OHPro assay. Values are expressed as the mean $\pm$ S.D. of quadruplicate assays. * P <0.05, *** P <0.001, vs IL-1 α . B: Cytotoxicity results of SKI306X and *Trichosanthes kirilowii* in rabbit cartilage explant culture. LDH assays were performed on day 7 and 14 media to assess viability of explanted tissue.

culture medium and the effect of SKI306X on expression of MMP-3 and -13. If rabbit cartilage collagen is degraded by MMPs, the activity of these enzymes should be detectable in these cultures. We therefore determined total MMPs activities and several selective MMPs activities in cartilage culture media using peptide cleavage analysis (Table 1). The quenched fluorescent substrates to total MMPs and specific quenched fluores-

cent substrates to MMP-1, -3, -8, and -13 were used to monitor the appearance of MMPs activities in the media of cartilage culture. The control cartilage cultures (without IL-1 α) showed negligible amount of activities at any time or during the first 2 weeks of culture with IL-1 α . However, there was a large increase in MMPs activities during the third week of IL-1 α stimulation, which correlated with the beginning of collagen dissolution. When

Fig. 3. Western immunoblotting analysis of rabbit cartilage explants culture medium. Cartilage slices were incubated for 2 h in DMEM containing specific concentration of SKI306X and *Trichosanthes kirilowii*. Then 5 ng/ml of IL-1 α was added to the medium and cultures were incubated for 15 days. Culture media were analyzed by Western blot using anti-rabbit proMMP-3 and anti-human proMMP-13 monoclonal antibody. A: SKI306X treatment, lane 1: control, lane 2: IL-1 α , lane 3: IL-1 α + 0.3 mg/ml SKI306X, lane 4: IL-1 α + 0.1 mg/ml SKI306X, lane 5: IL-1 α + 0.03 mg/ml SKI306X, lane 6: IL-1 α + 10 μ M dexamethasone. B: *Trichosanthes kirilowii* treatment, lane 1: control, lane 2: IL-1 α , lane 3: IL-1 α + 0.3 mg/ml *Trichosanthes kirilowii*, lane 4: IL-1 α + 0.1 mg/ml *Trichosanthes kirilowii*, lane 5: IL-1 α + 0.03 mg/ml *Trichosanthes kirilowii*, lane 6: IL-1 α + 10 μ M dexamethasone.

SKI306X and *Trichosanthes kirilowii* at concentration of 0.3 mg/ml were introduced into the day-15 medium, the above proteolytic activity of total MMPs was reduced significantly and the proteolytic activities of MMP-3 and MMP-13 were also attenuated over 50% in the culture medium up to 2 weeks. On the other hand, SKI306X and *Trichosanthes kirilowii* had no effect on the other MMPs such as MMP-1 and MMP-8. This result showed that the inhibitory effect of SKI306X on total expressed MMPs is due to expression inhibition of MMP-3 and -13. Diclofenac, rofecoxib, and dexamethasone did not decrease total MMPs activity and MMP-3 and MMP-13 activity (data not shown).

SKI306X effects on proenzyme MMP-3 to APMA-mediated activation

In Western blotting, two bands are detected in the lane corresponding to MMP-3. One of these bands may be the proenzyme form and the other is the active form of MMP-3, since the MMPs enzymes exist as a proenzyme form and an active form. Therefore, we investigated the effect of SKI306X on the activation procedure of MMP-3. To study the interactions between proenzyme MMP-3, SKI306X, and *Trichosanthes kirilowii*, APMA-mediated activation of proMMP-3 was carried out. Recombinant human proenzyme MMP-3 was incubated with APMA including SKI306X and *Trichosanthes kirilowii* and was analyzed by using gelatin zymography. MMP-3 is secreted as a 57- and 59-kDa proenzyme and can be activated in vitro by oronomer-

Table 1. Cumulative MMPs activities in control medium and IL-1 α -stimulated culture medium of rabbit cartilage explants

A. Cumulative total MMPs activity

Week of culture	Relative fluorescence			
	Control	IL-1 α	SKI306X (0.3 mg/ml)	<i>Trichosanthes kirilowii</i> (0.3 mg/ml)
1	0.71 $\pm$ 0.1	1.98 $\pm$ 0.2	1.75 $\pm$ 0.2	0.02 $\pm$ 0.01
2	1.76 $\pm$ 0.2	7.95 $\pm$ 2.5	5.58 $\pm$ 0.5	4.99 $\pm$ 0.2
3	2.72 $\pm$ 0.2	29.89 $\pm$ 8.5	18.04 $\pm$ 5.1	13.86 $\pm$ 1.5

B. Cumulative MMP-3, MMP-13 activity

Week of culture	Relative fluorescence							
	Control		IL-1 α		SKI306X (0.3 mg/ml)		<i>Trichosanthes kirilowii</i> (0.3 mg/ml)	
	MMP-3	MMP-13	MMP-3	MMP-13	MMP-3	MMP-13	MMP-3	MMP-13
1	0.32 $\pm$ 0.1	0.25 $\pm$ 0.1	1.25 $\pm$ 0.1	0.89 $\pm$ 0.1	0.68 $\pm$ 0.1	1.03 $\pm$ 0.1	0.1 $\pm$ 0.1	0.36 $\pm$ 0.1
2	0.75 $\pm$ 0.1	0.69 $\pm$ 0.1	5.55 $\pm$ 1.2	6.54 $\pm$ 1.5	2.58 $\pm$ 0.2	3.06 $\pm$ 0.2	1.32 $\pm$ 0.1	1.69 $\pm$ 0.8
3	1.84 $\pm$ 0.2	1.66 $\pm$ 0.1	17.21 $\pm$ 2.6	15.29 $\pm$ 2.8	10.98 $\pm$ 2.5	11.99 $\pm$ 2.1	9.56 $\pm$ 3.1	8.09 $\pm$ 3.4

Results are expressed as relative fluorescence of product produced per explants.

Fig. 4. Gelatin zymography analysis of APMA-mediated activation of proenzyme MMP-3. The purified human recombinant proenzyme MMP-3 was activated with APMA in the presence of herbal extracts and dexamethasone. The inhibitory effects on activation by herbal extracts and dexamethasone were analyzed by gelatin zymography. Lane 1: human recombinant proenzyme MMP-3 (50 ng), lane 2: APMA-treated human recombinant MMP-3, lane 3: 0.3 mg/ml SKI306X treatment, lane 4: 0.3 mg/ml *Trichosanthes kirilowii* treatment, lane 5: 10 μ M dexamethasone treatment.

Table 2. Inhibitory effects of SKI306X on MMPs activity (IC_{50})

Materials	MMPs IC_{50}			
	SKI306X (mg/ml)	<i>Trichosanthes kirilowii</i> (mg/ml)	BB94 (μ M)	Dexamethasone (μ M)
MMP-1	86 ± 12.15	0.86 ± 0.32	0.45	>1000
MMP-3	>1000	0.63 ± 0.60	0.84	>1000
MMP-8	125 ± 4.28	0.89 ± 0.19	0.75	>1000
MMP-13	69 ± 18.31	1.02 ± 0.45	0.55	>1000

MMPs activity was assayed by a quenched fluorescent substrate assay.

curials (e.g., 4-aminophenylmercuric acetate, APMA) via intermediate forms of 45-kDa active MMP-3 enzyme. Gelatin zymography allows detection of the 57-, 59-kDa pro form, 45-kDa active form, and 28-kDa degenerative form of MMP-3. SKI306X and *Trichosanthes kirilowii* inhibited activation of proMMP-3 to active MMP-3 at a concentration of 0.3 mg/ml (Fig. 4). We could not find any commercially available inhibitors of MMP-3 activation, so we tested only SKI306X and *Trichosanthes kirilowii*. However, these results can suggest that SKI306X and *Trichosanthes kirilowii* could have inhibitory effects on the activation procedure of proenzyme MMP-3 by APMA involved in cartilage degradation.

SKI306X effects on MMPs enzymes activity

To elucidate direct inhibitory effects of SKI306X on MMPs activities, human recombinant MMP-1, MMP-3, MMP-8, and MMP-13 were used. MMPs activities were analyzed by a peptide cleavage assay using a quenched fluorescent peptide as a substrate. SKI306X inhibited most of the MMPs activities (IC_{50} values: MMP-1, 86 ± 12 mg/ml; MMP-8, 125 ± 4 mg/ml; and MMP-13, 69 ± 18 mg/ml) at very high concentration, but not MMP-3. *Trichosanthes kirilowii* significantly inhibited MMPs activities including MMP-3 at mg/ml concentration (IC_{50} values: MMP-1, 0.86 ± 0.32 mg/ml; MMP-3, 0.63 ± 0.6 mg/ml; MMP-8, 0.89 ± 0.19 mg/ml; and MMP-13, 1.02 ± 0.45 mg/ml) (Table 2). SKI306X and *Trichosanthes kirilowii* showed activity an inhibitory effect on MMPs in only high doses. Activity inhibition

of MMPs by SKI306X is not a major mechanism for inhibition of collagen degradation.

Discussion

OA is a common joint disease that is also a major cause of disability in humans. It occurs through several complex mechanisms such as progressive erosion of cartilage, PG degradation, and disruption of the collagen network, leading to a progressive destruction of joints and functional loss. Increased catabolism of the matrix collagen network, collagen and aggrecan (cartilage PG aggregate), is the principal pathological process that leads to the degeneration of cartilage in OA (18). In general, the erosion of cartilage in OA is initially caused by a decrease in the PG content and then followed by the destruction of collagen matrix. Some studies suggested that investigations on cartilage degradation should include the monitoring of both collagen and PG catabolism (1, 19).

Therefore, we focused on the protective effects of SKI306X on the release of PG and collagen in the rabbit cartilage explants cultures and its mechanisms. In previous studies, SKI306X was demonstrated to have protective effects in rabbit articular cartilage and inhibitory effects on GAG release (9). Our co-workers found that SKI306X can inhibit aggrecanases activity in bovine and porcine explant cultures. Through these mechanism, SKI306X could exert PG degradation inhibition (submitted). The present study has demonstrated that IL-1 α -induced degradation of the matrix

network including PG and collagen was inhibited by the herbal extracts and has investigated the mechanisms of cartilage protective action by the drug.

Previous investigators showed that IL-1 α induced degradation of more than 60% collagen after 15 to 25 days in bovine cartilage explants and rabbit cartilage explants (20, 21). In this study, collagen degradation by IL-1 α was significantly increased after day 17. This increase was, however, significantly reduced by treatment with SKI306X. To investigate the mechanism of collagen degradation by IL-1 α , we focused on MMP enzymes. We found that IL-1 α stimulated expressions of MMP-3 and MMP-13 in rabbit cartilage explants culture.

The MMPs acting collectively have the capacity to degrade all components of extracellular matrix including PG and collagen. In human OA, MMPs are suggested to play key roles in the destruction of cartilage. It also has been shown that MMP-1, MMP-3, MMP-8, and MMP-13 are produced and secreted by several cell types including the chondrocyte (22, 23). This chondrocyte, indicated by a recent work, may have a central role in cartilage matrix destruction in OA (24). Poorly regulated MMPs activity and expression have been implicated in a number of diseases including RA, OA, cancer, periodontal disease, and inflammatory bowel disease (25).

Several members of the MMP family are known to be involved in OA, especially collagenases (MMP-1, MMP-8, MMP-13) and stromelysin-1 (MMP-3). Although it appears that stromelysins and gelatinases do not play a significant role in the destruction of articular cartilage, the elusive aggrecanase is still being actively pursued and some scholars have recently published data that support the view that a collagenase-like enzyme such as stromelysin-1 may have a role in the degradation of the PG core. The role of the collagenases, on the other hand, is now fairly well established. MMP-1, -8, and -13 have all been found in cartilage and have all been implicated in the degradation of the type II collagen in the cartilage matrix. It is known that MMP-3 and MMP-13 are produced and secreted by several cell types, including the chondrocyte. Recent reports have indicated that the chondrocyte itself may have a central role in cartilage matrix destruction in OA (26–30).

These facts suggesting the critical roles of MMPs in collagen degradation and our finding that SKI306X prevents collagen degradation motivated us to investigate the effects of SKI306X on the MMPs. The effects were observed by the following 4 parameters: 1) the expression inhibition of MMPs induced by IL-1 α in supernatant from rabbit cartilage explant culture, 2) the expression inhibition by activities of MMPs in the supernatant from rabbit cartilage explant culture, 3) con-

version inhibition of purified human recombinant proenzyme MMPs to active form MMPs in a cell free in vitro system, and 4) enzymatic activities inhibition of MMPs in a cell free in vitro system.

SKI306X's three oriental component herbs, *Clematis mandshurica*, *Trichosanthes kirilowii*, and *Prunella vulgaris*, have been widely used for the treatment of inflammatory diseases such as lymphadenitis and arthritis in Far East Asia and are known to have multi-functions such as anti-inflammation, immunomodulation, and the activation of blood microcirculation (7, 8). Each herb has been found to have different effects. In this study, we showed that SKI306X and *Trichosanthes kirilowii* are effective inhibitors of cartilage degradation at the same concentration. We suggest that the effect of SKI306X on cartilage protection could be due to *Trichosanthes kirilowii*. It significantly reduces PG and collagen loss.

To investigate the mode of action of SKI306X and *Trichosanthes kirilowii* in the cartilage protective effect, we tested changes in the level of several MMPs enzymes including MMP-3 and MMP-13. It was seen at the protein level by Western blot analysis and fluorogenic substrate assay. SKI306X and *Trichosanthes kirilowii* efficiently inhibited the expression of MMP-3 and -13, but SKI306X and *Trichosanthes kirilowii* did not inhibit MMPs activity at low to moderate concentrations (showed the inhibitory effect at high concentrations). In addition, SKI306X and *Trichosanthes kirilowii* inhibited the activation of proenzyme MMP-3 to active MMP-3. We used dexamethasone as a positive control drug. Dexamethasone had an inhibitory effect on collagen degradation in rabbit cartilage explant culture and the expression inhibition of MMPs without suppressing MMPs activity at high concentration. Rofecoxib, a COX-2 inhibitor, had no effects in any of the experiments.

In summary, SKI306X has the ability to inhibit collagen degradation and this effect is due to inhibition of MMP-3 and -13 expression according to results of Western blotting and fluorogenic substrate assay in rabbit cartilage explant culture without inhibiting activity of MMPs. It is also due to inhibition of MMP-3 activation. The cartilage protective effects of SKI306X is due to *Trichosanthes kirilowii*.

Our results thus suggest that SKI306X inhibits IL-1 α -induced matrix catabolism by suppressing expressions and activation processes of MMPs enzymes that degrade the cartilage matrix.

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