



Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: [www.elsevier.com/locate/jethpharm](http://www.elsevier.com/locate/jethpharm)



## *Panax ginseng* C.A. Meyer modulates the levels of MMP3 in S12 murine articular cartilage cell line

Joon-Shik Shin<sup>a</sup>, Namhee Park<sup>b</sup>, Jehyeon Ra<sup>c</sup>, Yangseok Kim<sup>b</sup>, Minkyu Shin<sup>b</sup>, Moochang Hong<sup>b</sup>, Sung-Hoon Kim<sup>d</sup>, Ha-Jeong Kwon<sup>c</sup>, Seon-Pyo Hong<sup>c</sup>, Jinju Kim<sup>c,\*</sup>, Hyunsu Bae<sup>a,\*\*</sup>

<sup>a</sup> *Jaseng Hospital of Oriental Medicine, Sinsa-dong, Kangnam-gu, Seoul 135-896, Republic of Korea*

<sup>b</sup> *Department of Physiology, College of Oriental Medicine, Kyung Hee University, #1 Hoeki-dong Dongdaemoon-gu, Seoul 130-701, Republic of Korea*

<sup>c</sup> *Department of Oriental Pharmaceutical Sciences, Kyung Hee East-West Pharmaceutical Research Institute, College of Pharmacy, Kyung Hee University,*

*#1 Hoeki-dong Dongdaemoon-gu, Seoul 130-701, Republic of Korea*

<sup>d</sup> *Department of Oriental Pathology, College of Oriental Medicine, Kyung Hee University, #1 Hoeki-dong Dongdaemoon-gu, Seoul 130-701, Republic of Korea*

### ARTICLE INFO

#### Article history:

Received 6 January 2009

Received in revised form 11 May 2009

Accepted 28 May 2009

Available online xxx

#### Keywords:

Cartilage degradation

*Panax ginseng* C.A. Meyer

Matrix metalloproteinases

Phospho-p38

### ABSTRACT

**Aim of the study:** The destruction of cartilage in patients with osteoarthritis occurs due to an imbalance between matrix synthesis and degradation. Cartilage degradation is induced by the activation of matrix metalloproteinases (MMPs). Therefore, this study was conducted to evaluate the cartilage protective effect of *Panax ginseng* C.A. Meyer (PG).

**Materials and methods:** S12 cells were treated with various concentrations of extract of PG and ginsenosides Rd and Rb<sub>3</sub> for 3 h, after which 10 ng/ml interleukin-1 $\beta$  (IL-1 $\beta$ ) was added to the culture media. The levels of MMP3 in the conditioned media were then evaluated using an enzyme-linked immunosorbent assay (ELISA). In addition, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to evaluate the mRNA expression of Type II Collagen and Pro-collagenase. Furthermore, Western blot analysis was performed to identify the roles that PG played in the ERK and p38 signaling pathways.

**Results:** The MMP3 secretion levels of S12 cells were significantly lowered in response to treatment with PG and ginsenosides Rd and Rb<sub>3</sub> at a concentration of 100  $\mu$ g/ml when compared to cells that were treated with IL-1 $\beta$ . In addition, PG induced the mRNA expression of Type II Collagen dose dependently. Furthermore, phosphorylated p38 and ERK were detected in S12 articular cartilage cell line that was treated with IL-1 $\beta$ . PG decreased the phosphorylation of p38, but PG did not exert any effect on phospho-ERK.

**Conclusions:** These findings indicate that PG and ginsenosides Rd and Rb<sub>3</sub> suppress MMP3 secretion and that ginsenosides Rd and Rb<sub>3</sub> are the major elements involved in the suppression of MMP3 by PG. Furthermore, the suppression of MMP3 by PG occurs via the inhibition of phospho-p38 activation. Therefore, PG may exert a protective effect against the cartilage degradation of OA.

© 2009 Published by Elsevier Ireland Ltd.

### 1. Introduction

Osteoarthritis (OA) is a common disease with an incidence that increases as age increases. Although the pathogenesis of OA is not yet well understood, it is known to be associated with synovial inflammation, degradation of articular cartilage and remodeling of the subchondral bone (Felson, 2004). The degenerative change

that occurs in the joints of OA patients is triggered by a series of biochemical events, including the production of the interleukin-1 $\beta$  (IL-1 $\beta$ ), which stimulates cartilage matrix degradation by inhibiting the production of Type II Collagen while up-regulating the production of matrix-degrading enzymes such as matrix metalloproteinases (MMPs) (Fernandes et al., 2002; Henrotin et al., 2006). Cartilage degradation is mediated by MMPs, and the increased release of MMP3 may be associated with the frequent development of OA (Lohmander et al., 1994) (Lin et al., 2004).

Current treatments for the management of OA are not curative and do not reverse the degenerative process of OA. Nonsteroidal anti-inflammatory drugs (NSAIDs) are often used for the treatment of OA, but their prolonged consumption is associated with serious adverse side effects such as cardiovascular risks (Henrotin et al., 2006) and gastrointestinal ulcerations (Long et al., 2001).

\* Co-corresponding author at: Department of Oriental Pharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Seoul 130-701, Republic of Korea. Tel.: +82 2 961 9437; fax: +82 2 968 0560.

\*\* Corresponding author at: Department of Physiology, College of Oriental Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea. Tel.: +82 2 961 9316; fax: +82 2 967 2080.

E-mail addresses: [shdwer@khu.ac.kr](mailto:shdwer@khu.ac.kr) (J. Kim), [hbae@khu.ac.kr](mailto:hbae@khu.ac.kr) (H. Bae).

Accordingly, the need for effective treatment modalities with fewer side effects has prompted OA patients to consider complementary approaches that allow them to control pain while improving their quality of life.

Nuclear factor of activated T cells (NFAT) transcription factors (NFATp) is a repressor of cartilage cell growth and differentiation. The S12 cell line was generated from the articular cartilage of NFATp<sup>-/-</sup> mutant mice primary cells (Ranger et al., 2000).

Jaseng-Ko is a prescription of Korean Oriental Medicine that is commonly used to control pain and improve function in OA patients. In this study, we screened the 6 species of Korean traditional medicine that comprise Jaseng-Ko for inhibitors of MMP3 in IL-1 $\beta$  treated S12 cell line, and we identified *Panax ginseng* C.A. Meyer (PG) as a most significant inhibitor about MMP3 of 6 ingredients of Jaseng-Ko in the S12 cell line. Therefore, we investigated the roles that PG plays in IL-1 $\beta$ -induced S12 cells.

## 2. Materials and methods

### 2.1. Preparation of Korean Oriental Medicines

Jaseng-Ko is comprised of *Rehmannia glutinosa* Libschitz var. *purpurea* Makino, *Achyranthes bidentata* Blume, *Panax ginseng* C.A. Meyer, *Cervi Pantotrichum Cornu*, *Asini Gelatinum*, and *Poria cocos* Wolf. The sprayed dried extracts of these 6 ingredients were purchased from the Sun Ten Pharmaceutical Company (Taipei, Taiwan) and subsequently screened. Jaseng-Ko is kindly provided from Jaseng Hospital of Oriental Medicine, Seoul, Korea. All dried extracts were deposited in the herbarium at the Department of Oriental Medicine in Kyung Hee University.

### 2.2. Reagents and antibodies

Gensenosides Rd and Rb<sub>3</sub> were purchased from LKT Laboratories (LKT Laboratories, Minnesota, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Invitrogen Corporation, California, USA) and fetal bovine serum (FBS) and antibiotics were purchased from Gibco (Gibco BRL, Maryland, USA). MTT (methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) was purchased from Promega (Promega Corporation, Wisconsin, USA). The following antibodies that were used for western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA): anti-phospho-ERK, anti-phospho-p38, anti-beta actin, anti-ERK, and anti-p38. In addition, MMP3 enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (R&D Systems, Minnesota, USA). Finally, mouse recombinant IL-1 $\beta$  was purchased from R&D Systems Inc. (R&D Systems, Minneapolis, MN, USA).

### 2.3. Cell culture

S12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing heat-inactivated 10% fetal bovine serum (FBS) and 100 units/ml of penicillin streptomycin (Gibco BRL, Maryland, USA). The S12 cell line is currently stocked at the Department of Physiology, College of Oriental Medicine in Kyung Hee University.

### 2.4. Treatment of S12 cells with IL-1 $\beta$ and Korean Oriental Medicines

S12 cells were placed in 48-well plates and then treated with 100  $\mu$ g/ml of several Korean Oriental Medicine extracts and screened for MMP3 secretion. In addition, S12 cells were placed in 24-well plates and treated with various concentrations of PG as well

as gensenoside Rd and Rb<sub>3</sub> for MMP3 secretion. After 3 h of pretreatment, 10 ng/ml IL-1 $\beta$  (R&D Systems, Minneapolis, USA) was added to the culture media, and the cells were then incubated at 37 °C for 3 additional days. The supernatants were then harvested by centrifugation, after which they were stored at -20 °C until assayed. The supernatants are currently stocked at the department of Oriental medicine in Kyung Hee University.

### 2.5. MTT assay

A Cell Titer 96™ non-radioactive cell proliferation assay (Promega Corporation, Wisconsin, USA) was used following the directions provided by the manufacturer. The cell viability was evaluated using tetrazolium compound MTT(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) based on its mitochondrial-dependent reduction to formazan. Specifically, well plates were seeded with cells at a density of 5 × 10<sup>3</sup> cells/well in a total volume of 100  $\mu$ l. The plates were then incubated overnight, after which the cells were treated with PG or gensenoside Rd or Rb<sub>3</sub> (0, 0.001, 0.01, 0.1, 1, 10, 100  $\mu$ g/ml). Cells in the control group were left untreated. The cells were then incubated for another 24 h at 37 °C under 5% CO<sub>2</sub>. Next, 20  $\mu$ l of MTT solution were added to each well and the plates were then incubated for an additional 2 h under the same conditions. After incubation, the formation of formazan was measured at 540 nm using an automated Titertek Multiscan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL). All data were expressed as the mean percentage of viable cells vs. the control.

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

The culture supernatants from the S12 cells that were harvested after 72 h of incubation were evaluated using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, flat-bottomed, 96-well plates were coated overnight at room temperature using coating buffer containing capture antibody of MMP3. Next, the wells were washed 3 times with PBS-T (PBS in 0.05% Tween-20), after which they were blocked with Reagent Diluent (PBS in 1% BSA) for 1 h at room temperature. The plates were then washed with the same wash buffer used above and subsequently blotted on a paper towel. Diluted samples of the culture media and standards were then added to the appropriate wells in triplicate, which were subsequently incubated for 2 h at room temperature. Next, the wells were washed 3 times, after which MMP3 detection antibody was added to each well and the plates were incubated for an additional 2 h at room temperature. The samples were then washed 3 times with PBS-T, after which streptavidin-HRP was added and incubated for 20 min at room temperature. The samples were then washed 3 times with PBS-T, and then tetramethylbenzidine substrate solution (Pharmingen, California, USA) was added. The color was then allowed to develop for 20 min in the dark, after which the reaction was quenched by stop solution (0.2 M H<sub>2</sub>SO<sub>4</sub>). Finally, the absorbance of the plates was measured at 450 nm using an automated Titertek Multiscan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL) and the sample concentrations were then determined by comparison of these values to those in a standard curve.

### 2.7. RT-PCR analysis of gene expression

To measure the gene expression of Type II Collagen, Procollagenase and GAPDH, S12 cells were cultured as described above and then serum-starved and treated with 10 ng/ml IL-1 $\beta$  for 24 h. The cells were then lysed in 500  $\mu$ l of Trizol® reagent (Invitrogen

Corporation, California, USA), after which the total RNA was isolated according to the manufacturer's protocols. The integrity of the RNA was then confirmed by denaturing agarose gel electrophoresis (data not shown), and the total RNA was quantified by determining the optical density at 260 nm (OD<sub>260</sub>). Next, the RNA (1 µg) was treated with DNAase and then added to a 20 µl RT reaction mixture comprised of 1× RT buffer, 1 mM deoxynucleotide mix, 60 µM primer, 20 units of RNAase inhibitor, and 10 units of RT (all obtained from Roche Applied Science, Germany). The reaction mixture was then incubated at 55 °C for 30 min.

PCR was performed using 1.25 units of Premix Taq™ (TaKaRa Biotechnology, Seoul, Korea) in a reaction mixture with a total volume of 25 µl. The reaction mixture was then subjected to the following conditions using a PTC 100-60HB RT-PCR thermal cycler (MJ Research, Inc., Massachusetts, USA) 35 cycles of 20 s of denaturation at 94 °C and 20 s of annealing at 55 °C for Type II Collagen and or Pro-collagenase 35 cycles of 20 s of denaturation at 94 °C and 20 s of annealing at 53 °C for GAPDH, followed by 1 min of elongation at 72 °C. The following sense and antisense Pro-collagenase and Type II Collagen primers (Genotech Corp., Seoul, Korea) were used: 5'-GCTGCTGACGCTGCTCATC-3' (sense) and 5'-GGTTCTCCTTTCTGCCCTT-3' (antisense) for Type II Collagen, 5'-ATCTGGCCACCTTCTTCTT-3' and (sense 5'-TTTCTCGGAGCCTGTCA-ATC-3' (antisense) for Pro-collagenase, 5'-CAGACTGTCCCGT-TTCCAT-3' (sense) and 5'-GGTGTGACTGCATCAAAGA-3' (antisense) and 5'-ACACATTGGGGGTAGGAACA-3' (antisense) for GAPDH, which was used as an internal control. An equal volume of each PCR product was analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining. Marker gene expression was normalized to the GAPDH expression for each sample and the signal intensity was quantified using the Gel Doc EQ (BIO-RAD Laboratories, Milan, Italy).

### 2.8. Western blot analysis

The proteins were extracted from the cells using lysis buffer PPO-PREP™ (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Briefly, the cell pellet was resuspended in 5 µl sample loading buffer (1 M Tris-HCL (Ph 6.8), 10% SDS, 50% glycerol, 1% bromophenol blue, and β-mercaptoethanol). Equal amounts of the cell lysates were then loaded into 4–12% sodium dodecyl sulfate–polyacrylamide gels and subjected to electrophoresis (SDS–PAGE, precast gel; Invitrogen Corporation, California, USA). The separated proteins were then transferred to nitrocellulose membranes (Protran, Schleicher & Schuell Bioscience, Dassel, Germany) for 2 h at 80 V using a transfer system. The membranes were then blocked in TBS-T (TBS with 0.05% Tween-20) containing 1% nonfat dry milk and 1% BSA for 1 h at room temperature. Next, the membranes were washed with TBS-T 3 times, after which they were incubated with primary antibodies against Anti-phospho-ERK, anti-phospho-p38, anti-beta actin, anti-ERK and anti-p38 (Santa Cruz Biotechnology, California, USA) overnight at 4 °C. Finally, the membranes were washed three times with TBS-T and then incubated with the corresponding secondary antibodies (Calbiochem, A Brand of EMD Biosciences, Inc., San Diego, USA) for 1 h, after which they were visualized using enhanced chemiluminescence (ECL; western blotting detection system; Amersham Biotech Inc., New Jersey, USA).

### 2.9. Statistical analysis

Statistical analysis was performed by Student's *t*-test and one-way ANOVA followed by post-hoc tests when necessary (Newman–Keuls Multiple Comparison Test). All data were analyzed using GraphicPad PRISM.

### 2.10. HPLC method for analysis of ginsenosides

A reversed-phase method of high performance-liquid chromatography-pulsed amperometric detection (RP-HPLC-PAD) was used for determination of ginsenosides (Kwon et al., 2008). The PAD system from the ICS-3000 series Dionex (Sunnyvale, CA, USA) was equipped with an Au-Flowcell containing a goldworking electrode and a solvent compatible cell containing an Ag/AgCl reference electrode. HPLC equipment, consisting of a Model Nanospace SI-2/3201 pump and a 3004 column oven, was purchased from Shiseido (Tokyo, Japan). Chromatographic separation was performed by using a Capcell Pak C18 column (150 mm × 1.0 mm I.D.; 3 µm, Shiseido, Tokyo, Japan). The potential waveform was as follows: E1 = -0.2 V (from 0.00 to 0.04 s); E2 = 0 V (from 0.05 to 0.21 s); E3 = +0.22 V (from 0.22 to 0.46 s); E4 = 0 V (from 0.47 to 0.56 s); E5 = -2 V (from 0.57 to 0.58 s); and E6 = +0.6 V (0.59 s). The mobile phase consisted of 10% (v/v) acetonitrile (solvent A) and 60% (v/v) acetonitrile (solvent B). The following procedure was employed: isocratic elution with A:B (80:20) for 8 min, linear gradient elution from A:B (80:20) to (62:38) from 8 to 10 min, isocratic elution with A:B (62:38) from 10 to 30 min, linear gradient elution from A:B (62:38) to (30:70) from 30 to 60 min, linear gradient elution from A:B (30:70) to (80:20) from 60 to 62 min and finally equilibration with A:B (80:20) from 62 to 70 min. The flow rate was 0.1 ml/min, and the separation temperature was 45 °C. A post-column delivery system of 200 mM sodium hydroxide with a flow rate of 0.4 ml/min was added to the RP-HPLC-PAD system. The mobile phase was made through being degassed by vacuum filtration after the mixture of water with acetonitrile on a daily basis, followed by sonication for 20 min before use. A post-column delivery system was purged to remove carbonate from the water with helium throughout the experiment. The data were controlled on a computer running the Chromeleon client program supplied by Dionex. The ginsenoside standards and PG were dissolved in 50% (v/v) acetonitrile/water and filtrated through a MFS 13 disposable syringe filter before being applied to RP-HPLC-PAD. The concentration of the internal standard, digitoxin, was 20 µg/ml for all analytes. The injection volume was 10 µl.

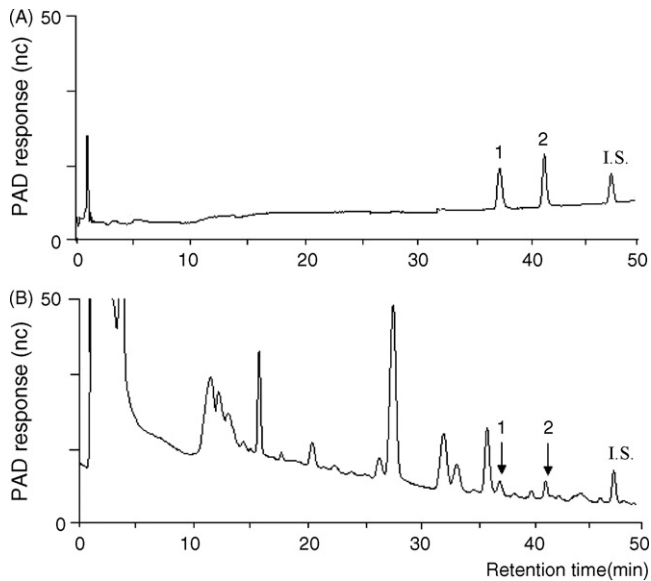
## 3. Results

### 3.1. Analysis of ginsenosides in PG

In order to determine the contents of ginsenoside Rd and Rb<sub>3</sub> in PG, we tested the standard ginsenosides. The linearity of detection was examined using four different standard solutions (1, 5, 10, and 20 µg/ml). A calibration curve was constructed by linear regression of the peak area ratio (analyte/internal standard) vs. analyte concentration. The linear equations of ginsenoside Rd and Rb<sub>3</sub> were  $y = 0.0837x + 0.2182$  and  $y = 0.0751x + 0.1041$ , respectively. The contents of ginsenoside Rd and Rb<sub>3</sub> in PG calculated by the linear equations were  $1.23 \pm 0.00$  and  $0.70 \pm 0.08$  mg/g, respectively. Fig. 1 shows the chromatograms of standard ginsenosides and PG.

### 3.2. Screening for MMP3 inhibition

Jaseng-Ko and its six ingredients, comprising Jaseng-Ko, were screened to identify inhibitors of MMP3 in IL-1β treated S12 cells. When we evaluated the levels of MMP3 in IL-1β treated cells after 3 days with or without treatment with Jaseng-Ko and each Korean Oriental Medicine treatment (100 µg/ml), Jaseng-Ko ( $*p < 0.05$ ), *Rehmannia glutinosa* Libschitz var. *purpurea* Makino ( $**p < 0.01$ ), *Panax ginseng* C.A. Meyer ( $***p < 0.001$ ) and *Poria cocos* Wolf ( $**p < 0.01$ ) were found to inhibit significantly the secretion of

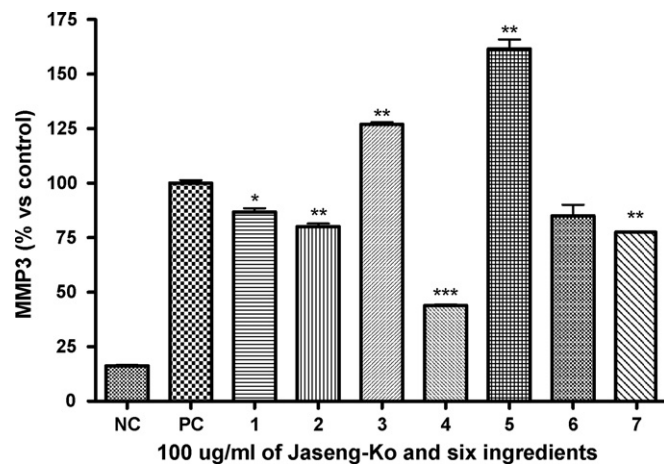


**Fig. 1.** Chromatograms of standard ginsenosides (A) and PG (B). Peaks: 1, ginsenoside Rb<sub>3</sub>; 2, ginsenoside Rd; I.S., digitoxin. Injected amounts: 200 ng of each ginsenoside (A) and 50 μg of PG (B).

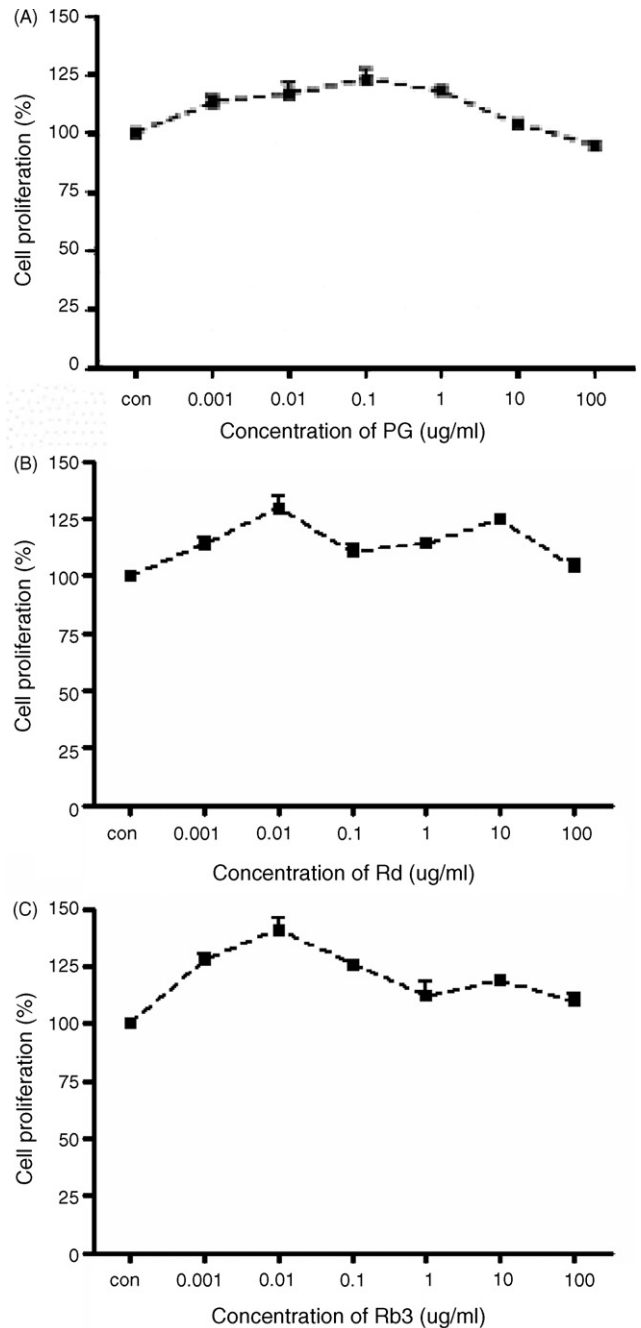
MMP3 in IL-1β treated S12 cells (Fig. 2). Of these, PG was selected for the subsequent assays.

### 3.3. Cell viability in response to treatment with PG and ginsenosides

The MTT assay was conducted to evaluate the cytotoxicity of PG, ginsenosides Rd and Rb<sub>3</sub> against S12 cells. These cells were treated with PG or ginsenosides Rd or Rb<sub>3</sub> (0, 0.001, 0.01, 0.1, 1, 10, 100 μg/ml). The results revealed that PG and ginsenoside Rd and Rb<sub>3</sub> exerted no significant cytotoxicity at any of the concentrations evaluated (Fig. 3).



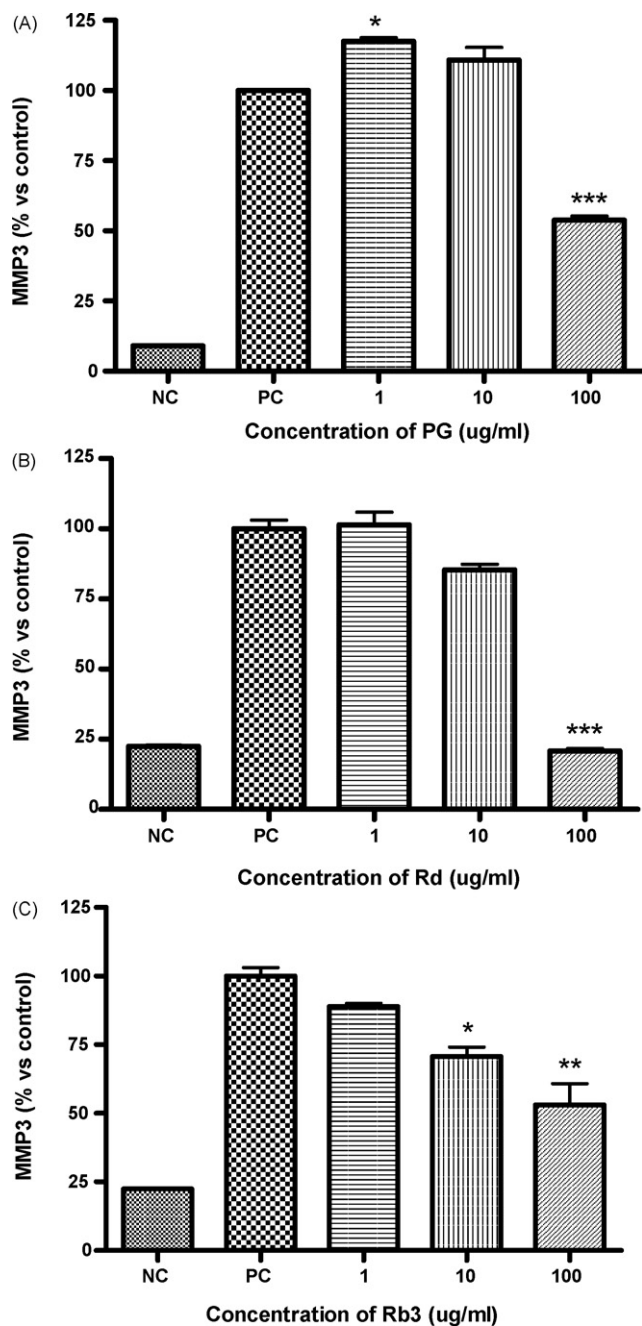
**Fig. 2.** Screening test of Korean Oriental Medicine against MMP3 inhibition in S12 cells by ELISA analysis. S12 cells were cultured in medium only (NC), medium containing 10 ng/ml IL-1β (PC), and medium containing 10 ng/ml IL-1β plus 100 μg/ml of Jaseng-Ko and each ingredient of Jaseng-Ko. NC: negative control, PC: positive control, 1: *Jaseng-Ko*, 2: *Rehmannia glutinosa* Libschitz var. *purpurea* Makino, 3: *Achyranthes bidentata* Blum, 4: *Panax ginseng* C.A. Meyer, 5: *CERVI PANTOTRICHUM CORNU*, 6: *Asini Gelatinum*, 7: *Poria cocos* Wolf. Statistical analysis was performed using Student's *t*-test; \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. PC. Data shown are the means ± S.E.M.



**Fig. 3.** Viability of S12 cells following exposure to PG, ginsenosides Rd and Rb<sub>3</sub>. S12 cells were cultured in medium only (con) or in medium containing different concentrations of PG (A), Rd (B) and Rb<sub>3</sub> (C). The viability of the cells was then determined by an MTT assay. Data shown are the means ± S.E.M.

### 3.4. PG and ginsenosides Rd and Rb<sub>3</sub> inhibit the secretion of MMP3

We utilized ELISA assay to evaluate the inhibition effects of IL-1β mediated MMP3 secretion exerted by treatment with various concentrations of PG and ginsenosides Rd and Rb<sub>3</sub>. A significant reduction (\*\**p* < 0.001) in the MMP3 secretion was observed in response to treatment with PG at the concentration of 100 μg/ml (Fig. 4A). In addition, the treatment with Rd (100 μg/ml) resulted in a significant reduction (\*\**p* < 0.001) in MMP3 levels when compared to the cells treated with IL-1β (PC) (Fig. 4B). Finally, treatment with Rb<sub>3</sub> at concentrations of 10 and 100 μg/ml resulted in a sig-

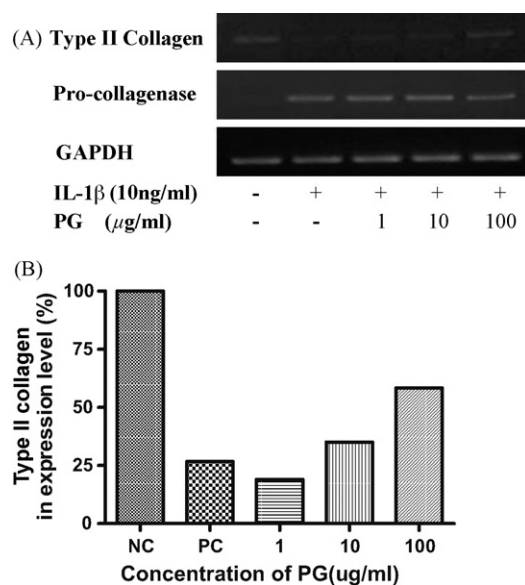


**Fig. 4.** Effect of PG, ginsenosides Rd and Rb<sub>3</sub> on MMP3 protein by ELISA analysis. S12 cells were cultured in medium only (NC), medium containing 10 ng/ml IL-1 $\beta$  (PC), or medium containing 10 ng/ml IL-1 $\beta$  plus different concentrations of PG ginsenosides Rd and Rb<sub>3</sub>. NC: negative control, PC: positive control, Statistical analysis was performed by one-way ANOVA followed by post-hoc test (Newman-Keuls Multiple Comparison Test); \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. positive control group. Data shown are the means  $\pm$  S.E.M.

nificant decrease in MMP3 levels (\* $p < 0.05$  and \*\* $p < 0.01$ ) when compared to cells treated with IL-1 $\beta$  (Fig. 4C).

### 3.5. Effects of PG on the induction of Type II Collagen mRNA in S12 cells

To determine if PG regulates the expression of mRNA involved in the degradation of cartilage, RT-PCR was conducted. The mRNA expression of Type II Collagen in S12 cells treated with IL-1 $\beta$  alone (PC) was down-regulated compare to none treated IL-1 $\beta$  (negative control; NC) The Type II Collagen mRNA expression was increased



**Fig. 5.** Effects of PG on Type II Collagen and Pro-collagenase mRNA by RT-PCR. S12 cells were cultured in medium only (NC), medium with 10 ng/ml IL-1 $\beta$  (PC), medium with 10 ng/ml IL-1 $\beta$  plus various concentrations of PG. NC: negative control, PC: positive control. Cells were incubated in IL-1 $\beta$  treated medium containing various concentrations (1, 10 and 100  $\mu$ g/ml) of PG. PG: *Panax ginseng* C.A. Meyer. GAPDH was used for loading control for normalization purpose. Type II Collagen and Pro-collagenase mRNA expressions (A), results of the densitometric analysis (B).

by treatment with PG at concentrations of 10 and 100  $\mu$ g/ml, and this increase was found to be dose-dependant (Fig. 5A and B). However, the effect of PG on the Pro-collagenase mRNA expression was not significantly altered comparing with cells treated with IL-1 $\beta$ .

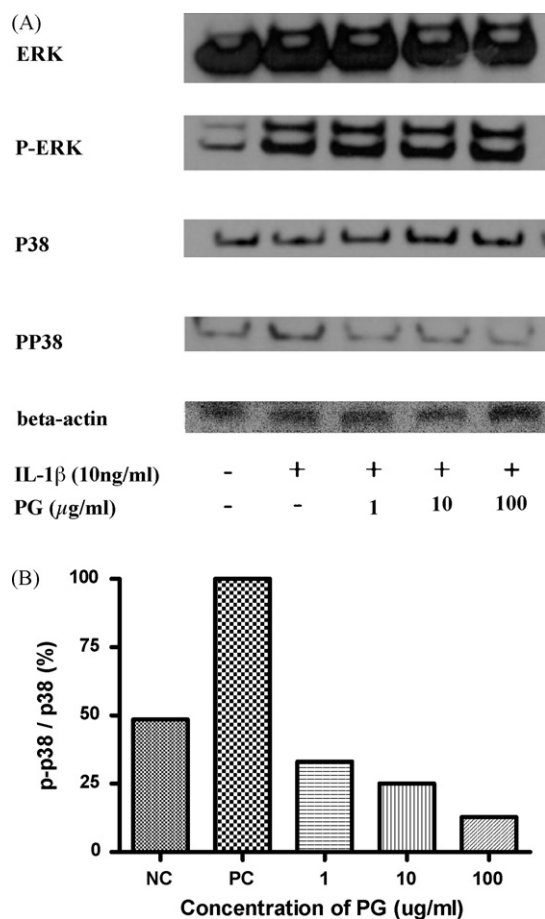
### 3.6. Effects of PG on the reduction of p38 phosphorylation in S12 cells

To identify the roles of the PG in ERK and p38 signaling pathways in S12 cells that were treated with 10 ng/ml IL-1 $\beta$ , western blot analysis was performed. Phosphorylated p38 and ERK were detected in S12 articular cartilage cell lines that were treated with IL-1 $\beta$ , and PG treatments inhibited the proportion of phosphorylated p38 form in total p38 from 67% to 87% comparing with cells treated IL-1 $\beta$  (PC), while the phosphorylation of ERK was not inhibited in S12 cells treated with IL-1 $\beta$  (Fig. 6).

## 4. Discussion

The MMP family members play a crucial role in tissue remodeling as well as in the destruction of cartilage and bone in arthritic joints (Burrage et al., 2006). In addition, the production of MMPs plays an important role in the development of OA in response to IL-1 $\beta$  stimulation (Mastbergen et al., 2002; Burrage et al., 2006). Among the various MMPs, MMP3 is capable of cleaving the Type II Collagen in cartilage, and in arthritic joints, degradation of Type II Collagen is usually excessive due to increased cleavage by MMPs (Billinghurst et al., 2000). The irreversible destruction of the cartilage that comprises synovial joints is characteristic of OA, and articular cartilage is made up of proteoglycans and Type II Collagen (Burrage et al., 2006).

PG has long been a popular medicinal plant. The demand for PG is associated with its reputation and empirical history PG, which have been supported by evidence-based data regarding its safety and effects from pre-clinical and clinical studies. Many studies have demonstrated the medicinal effects of PG and ginsenosides which



**Fig. 6.** Effects of PG on ERK and p38 phosphorylation by Western blot analysis. S12 cells were cultured in medium only (NC), medium with 10 ng/ml IL-1 $\beta$  (PC), medium with 10 ng/ml IL-1 $\beta$  plus various concentrations of PG. NC: negative control, PC: positive control. Cells were incubated in IL-1 $\beta$  treated medium containing various concentrations (1, 10 and 100  $\mu$ g/ml) of PG. PG: *Panax ginseng* C.A. Meyer. Beta actin was used for loading control for normalization purpose. Expression of total ERK, phosphor-ERK, p38 and phosphor-p38 (A), results of the densitometric analysis (B).

are constituents of ginseng species (Sung et al., 2000; Kim et al., 2003; Sengupta et al., 2004; Cheng et al., 2005; Nam et al., 2005; Tian et al., 2005; Radad et al., 2006; Rhule et al., 2008); however, to date, no studies have been conducted to evaluate the efficacy of PG on OA treatments. Our screening test about inhibitory effect against MMP3 in IL-1 $\beta$  treated S12 cells revealed that treatment with PG significantly inhibited IL-1 $\beta$  mediated MMP3 (Fig. 2). And treatment with 100  $\mu$ g/ml PG or ginsenosides Rd and Rb<sub>3</sub> significantly inhibited IL-1 $\beta$  mediated MMP3 (Fig. 4). Furthermore, the results of the present study demonstrated that PG, Rd and Rb<sub>3</sub> did not exert any cytotoxicity against S12 cells (Fig. 3). Taken together, these findings indicate that PG, Rd and Rb<sub>3</sub> suppress MMP3 secretion and that ginsenosides Rd and Rb<sub>3</sub> are the primary factors of PG responsible for the suppression of MMP3.

In this study, additional experiments were conducted to examine the mRNA levels of cartilage destruction in relation to the pathological determinants, Type II Collagen and Pro-collagenase, which are key control points in the breakdown of cartilage collagen (Milner et al., 2001; Ishikawa et al., 2004). IL-1 $\beta$  stimulates cartilage matrix degradation by inhibiting the production of Type II Collagen and increasing the level of collagenase (Wang et al., 1997; Fernandes et al., 2002). Therefore, in our experimental systems, the mRNA expression of Type II Collagen in S12 cells treated with IL-1 $\beta$  was down-regulated, and also Pro-collagenase mRNA expressions was up-regulated. And the Type II Collagen expression was found to

increase in IL-1 $\beta$  stimulated S12 cells that were treated with PG (10 and 100  $\mu$ g/ml), and this increase was found to be dose-dependant (Fig. 5A and B). However, PG could not show the decreasing effect on the Pro-collagenase mRNA expressions in IL-1 $\beta$  stimulated S12 cells.

In OA, the increase of MMP3 and the decrease of Type II Collagen are considered to contribute to degradation of the cartilage matrix. Therefore, the down-regulation of MMP3 and the up-regulation Type II Collagen would be reasonable therapeutic targets for the treatment of OA. Hence, our results suggest that PG has the potential to protect against cartilage degradation through regulation of MMP3 and Type II Collagen.

The induction of MMPs in response to treatment with IL-1 $\beta$  is known to be mediated via ERK and p38 (Kelley et al., 2007); therefore, we also investigated the effects of PG on the MAPK p38 or ERK signaling pathways of S12 cells that were treated with IL-1 $\beta$ . Phosphorylated p38 and phospho-ERK were detected in S12 cells in response to the induction of IL-1 $\beta$ . However, the phospho-p38 of IL-1 $\beta$  treated cells decreased in response to PG treatment, while no change in phospho-ERK was observed in response to PG treatment (Fig. 5).

In conclusion, PG suppressed the secretion of MMP3 in IL-1 $\beta$  stimulated S12 cells. Furthermore, PG increased the production of Type II Collagen in IL-1 $\beta$  stimulated S12 cells. Moreover, treatment with ginsenosides Rd and Rb<sub>3</sub> resulted in a significant reduction in MMP3 secretion in IL-1 $\beta$  treated S12 cells. Based on these results, it is likely that the ginsenosides Rd and Rb<sub>3</sub> are the constituents of PG that are responsible for the suppression of MMP3 by PG. In addition, the results of this study indicate that the effects of PG likely occur via the p38 signaling pathway. Therefore, PG is a potential therapeutic target for the modulation of collagen degradation that occurs in cases of osteoarthritis.

## Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. R13-2007-00000-0).

## References

- Billingham, R.C., Wu, W., Ionescu, M., Reiner, A., Dahlberg, L., Chen, J., van Wart, H., Poole, A.R., 2000. Comparison of the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1 and the selective inhibition of type II collagen cleavage by collagenase. *Arthritis and Rheumatism* 43, 664–672.
- Burrage, P.S., Mix, K.S., Brinckerhoff, C.E., 2006. Matrix metalloproteinases: role in arthritis. *Frontiers in Bioscience* 11, 529–543.
- Cheng, Y., Shen, L.H., Zhang, J.T., 2005. Anti-amestic and anti-aging effects of ginsenoside Rg1 and Rb1 and its mechanism of action. *Acta Pharmacologica Sinica* 26, 143–149.
- Felson, D.T., 2004. An update on the pathogenesis and epidemiology of osteoarthritis. *Radiologic Clinics of North America* 42, 1–9, v.
- Fernandes, J.C., Martel-Pelletier, J., Pelletier, J.P., 2002. The role of cytokines in osteoarthritis pathophysiology. *Biorheology* 39, 237–246.
- Henrotin, Y.E., Deberg, M.A., Crielgaard, J.M., Piccardi, N., Msika, P., Sanchez, C., 2006. Avocado/soybean unsaponifiables prevent the inhibitory effect of osteoarthritic subchondral osteoblasts on aggrecan and type II collagen synthesis by chondrocytes. *The Journal of Rheumatology* 33, 1668–1678.
- Ishikawa, T., Nishigaki, F., Christgau, S., Noto, T., Mo, J., From, N., Minoura, K., Hirayama, Y., Ohkubo, Y., Mutoh, S., 2004. Cartilage destruction in collagen induced arthritis assessed with a new biochemical marker for collagen type II C-telopeptide fragments. *The Journal of Rheumatology* 31, 1174–1179.
- Kelley, M.J., Rose, A.Y., Song, K., Chen, Y., Bradley, J.M., Rookhuizen, D., Acott, T.S., 2007. Synergism of TNF and IL-1 in the induction of matrix metalloproteinase-3 in trabecular meshwork. *Investigative Ophthalmology & Visual Science* 48, 2634–2643.
- Kim, N.D., Kim, E.M., Kang, K.W., Cho, M.K., Choi, S.Y., Kim, S.G., 2003. Ginsenoside Rg3 inhibits phenylephrine-induced vascular contraction through induction of nitric oxide synthase. *British Journal of Pharmacology* 140, 661–670.
- Kwon, H.J., Jeong, J.S., Lee, Y.M., Hong, S.P., 2008. A reversed-phase high-performance liquid chromatography method with pulsed amperometric detection for the determination of glycosides. *Journal of Chromatography A* 1185, 251–257.

- Lin, P.M., Chen, C.T., Torzilli, P.A., 2004. Increased stromelysin-1 (MMP-3), proteoglycan degradation (3B3- and 7D4) and collagen damage in cyclically load-injured articular cartilage. *Osteoarthritis Research Society* 12, 485–496.
- Lohmander, L.S., Roos, H., Dahlberg, L., Hoerrner, L.A., Lark, M.W., 1994. Temporal patterns of stromelysin-1, tissue inhibitor, and proteoglycan fragments in human knee joint fluid after injury to the cruciate ligament or meniscus. *Journal of Orthopaedic Research* 12, 21–28.
- Long, L., Soeken, K., Ernst, E., 2001. Herbal medicines for the treatment of osteoarthritis: a systematic review. *Rheumatology (Oxford)* 40, 779–793.
- Mastbergen, S.C., Lafeber, F.P., Bijlsma, J.W., 2002. Selective COX-2 inhibition prevents proinflammatory cytokine-induced cartilage damage. *Rheumatology (Oxford)* 41, 801–808.
- Milner, J.M., Elliott, S.F., Cawston, T.E., 2001. Activation of procollagenases is a key control point in cartilage collagen degradation: interaction of serine and metalloproteinase pathways. *Arthritis and Rheumatism* 44, 2084–2096.
- Nam, M.H., Kim, S.I., Liu, J.R., Yang, D.C., Lim, Y.P., Kwon, K.H., Yoo, J.S., Park, Y.M., 2005. Proteomic analysis of Korean ginseng (*Panax ginseng* C.A. Meyer). *Journal of Chromatography* 815, 147–155.
- Radad, K., Gille, G., Liu, L., Rausch, W.D., 2006. Use of ginseng in medicine with emphasis on neurodegenerative disorders. *Journal of Pharmacological Sciences* 100, 175–186.
- Ranger, A.M., Gerstenfeld, L.C., Wang, J., Kon, T., Bae, H., Gravallesse, E.M., Glimcher, M.J., Glimcher, L.H., 2000. The nuclear factor of activated T cells (NFAT) transcription factor NFATp (NFATc2) is a repressor of chondrogenesis. *The Journal of Experimental Medicine* 191, 9–22.
- Rhule, A., Rase, B., Smith, J.R., Shepherd, D.M., 2008. Toll-like receptor ligand-induced activation of murine DC2.4 cells is attenuated by Panax notoginseng. *Journal of Ethnopharmacology* 116, 179–186.
- Sengupta, S., Toh, S.A., Sellers, L.A., Skepper, J.N., Koolwijk, P., Leung, H.W., Yeung, H.W., Wong, R.N., Sasisekharan, R., Fan, T.P., 2004. Modulating angiogenesis: the yin and the yang in ginseng. *Circulation* 110, 1219–1225.
- Sung, J., Han, K.H., Zo, J.H., Park, H.J., Kim, C.H., Oh, B.H., 2000. Effects of red ginseng upon vascular endothelial function in patients with essential hypertension. *The American Journal of Chinese Medicine* 28, 205–216.
- Tian, J., Fu, F., Geng, M., Jiang, Y., Yang, J., Jiang, W., Wang, C., Liu, K., 2005. Neuroprotective effect of 20(S)-ginsenoside Rg3 on cerebral ischemia in rats. *Neuroscience Letters* 374, 92–97.
- Wang, A.Z., Wang, J.C., Fisher, G.W., Diamond, H.S., 1997. Interleukin-1beta-stimulated invasion of articular cartilage by rheumatoid synovial fibroblasts is inhibited by antibodies to specific integrin receptors and by collagenase inhibitors. *Arthritis and Rheumatism* 40, 1298–1307.