Effect of Betula platyphylla var. japonica on Proteoglycan Release, Type II Collagen Degradation, and Matrix Metalloproteinase Expression in Rabbit Articular Cartilage Explants

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Osteoarthritis (OA) is a degenerative joint disease characterized by the progressive loss of articular cartilage, subchondral bone remodeling, spur formation, synovial inflammation, and in particular, the degradation of proteoglycan and collagen. The integrity of these macromolecules is vital to cartilage and joint function.1)

Proteoglycan is a component of the articular cartilage extracellular matrix, providing it with many of its characteristic physicochemical properties.2) The carbohydrate component of aggrecan, which constitutes at least 90% of its molecular mass, consists of many long keratin sulfate, chondroitin sulfate, and glycosaminoglycan (GAG) chains covalently linked to a core protein.3) The contribution of these GAG polymers to cartilage function can be divided into two categories: firstly, maintenance of hydration, a property dependent on their polyanionicity, which engenders in cartilage the ability to withstand compression, and secondly, interactions with other macromolecules that depend on the specific distribution of the negative charges of GAG and its carbohydrate backbone conformation.4,5) Thus, the importance of these proteoglycans is clear. However, neither their specific role nor the mechanisms regulating their synthesis are fully understood.

Collagen is another component of articular cartilage, and it is present primarily as type II collagen.6) It plays a role in maintaining the integrity of the cartilage matrix and allows proteoglycan to be held in the matrix.7) Healthy cartilage maintains a dynamic equilibrium between processes that produce and processes that degrade matrix components. It is thought that this equilibrium is disturbed in OA, and that matrix-component degradative processes dominate, leading to an increased loss of the matrix. According to in vitro studies, collagen is much less readily released than proteoglycan, but collagen degradation results in the irreversible loss of structural integrity.8) There is circumstantial in vitro and in vivo evidence indicating a significant role for matrix metalloproteinases (MMPs) in cartilage degradation in arthritis.9)

MMPs can be classified into four subgroups: collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10, -11), gelatinases (MMP-2, -9), and membrane-type MMPs.10) MMPs are synthesized in response to cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α, which is thought to be involved in the pathogenesis of arthritis.10—13) MMPs are released as inactive proenzyme forms and are then activated by limited proteolysis of the propeptide domain by plasmin, kallikrein, or trypsin.14,15) 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 the degradation of these proteins in cartilage.15,16) MMP-13 is expressed by normal and osteoarthritic chondrocytes, and has been localized to both rheumatoid and osteoarthritic cartilage.17) MMP-13 is the most efficient collagenase against type II collagen, suggesting it plays an important role in cartilage collagen turnover.18,19)

Many cartilage protective agents have been developed from natural products, and they have resulted in the development of treatments for intractable diseases such as arthritis and inflammation.20,21) Recently, the search for potential cartilage protective agents has been pursued intensively, with promising clinical results, because there is renewed interest in the treatment of osteoarthritis in Oriental medicine. Betula platyphylla var. japonica (B. platyphylla) is one of these nat-
ural products, and is widely distributed in Korea, Japan, China, Sahalin, and Siberia. The bark of *Betula platyphylla* has been used in folk medicine for the treatment of arthritis, cancer, nephritis, dermatitis, poisoning, and chronic bronchitis. It has been reported that an extract of *Betula platyphylla* has antioxidant and anticancer activity.22)

The present study therefore investigates the cartilage-protective effects and mechanism of *Betula platyphylla* on rabbit articular cartilage.

**MATERIALS AND METHODS**

**Preparation of Betula platyphylla var. japonica Extract**

*Betula platyphylla* var. *japonica* was obtained from Kyung Hhee Oriental Medical Center, which was kindly confirmed by Nam-je Kim for quality control. The *Betula platyphylla* var. *japonica* was incubated in 50% (v/v) ethanol–water at room temperature for 24 h. The extract was then filtered and concentrated under low pressure using a vacuum rotary evaporator (Eyela, Japan). The remaining residue was condensed 5% CO2/95% air incubator to stabilize them. The complete medium and incubated for 1—2 d at 37 °C in a humidified 5% CO2/95% air incubator to stabilize them. The complete medium was replaced with basal medium (DMEM supplemented with 2.5% FBS, 10 mM HEPES, Tris base), and protein expression was determined using the Sircol Collagen Assay (Biocolor Ltd., Valley Business Center, Northern Ireland). Samples were reacted with Sirius red dye containing sulfonic acid for 30 min at room temperature. The reaction mixture measured optical density at 540 nm. The percentage of recovery was calculated from the absorbance of the sample relative to that of the standard. Collagen release (%)=sample treated cartilage—IL-1α treated cartilage ×100.

**RT-PCR Analysis of Gene Expression** RNA was prepared using Trizol® reagent (Invitrogen Corporation, CA, U.S.A.). Reverse transcription of 1 μg of total RNA was carried out for 60 min at 42 °C and then for 15 min at 72 °C using a system for RT-PCR (TaKaRa Biotechnology, Seoul, Korea), which contained RT buffer, oligo(dT) 12-mer, 10 mM dNTP, 0.1 mM dithiothreitol, reverse transcriptase, and RNase inhibitor. PCR using specific primers for each cDNA was carried out in a PCR reaction volume of 10 μl (as supplied by TaKaRa, Korea), supplemented with 2.5 units of TaKaRa Taq™ 1.5 mM each dNTP, 1×PCR buffer, and 20 pmol of each primer. Amplification reactions were performed using the following primers and protocol: MMP-3 forward, 5′-ATGGACCTTCTCCAGCAA-3′; reverse, 5′-TCATTATGGCATCAGCTC-3′; MMP-13 forward, 5′-AGAAGCATGCGCACTTCTAC-3′; reverse, 5′-TAAAAA GA CTCGCCCATCAA-3′; GAPDH: forward, 5′-GCTCTCAGAACATCACTCTGCC-3′; reverse, 5′-CTGGTCATACCGAGGAAATGAGCT-3′. An equal volume from each PCR was analyzed by 1.5% agarose gel electrophoresis, and ethidium bromide-stained PCR products were evaluated. Signal intensity was quantified with the Gel Doc EQ (BIO-RAD Laboratories, Milan, Italy).

**Western Blotting Analysis** MMPs in the culture medium were analyzed by Western blotting. Medium samples were boiled for 5 min with 2.5% mercaptoethanol and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 4—12% gradient gels under reducing conditions. Membranes were blocked with 5% nonfat dry milk in TBS-T (0.05% Tween 20, 138 mM NaCl, and 25 mM Tris base), and protein expression was determined using guineapig anti-rabbit MMP-3 or mouse anti-rabbit MMP-13 antibody (Calbiochem, CA, U.S.A.) at a dilution of 1:500 for 24 h. A horseradish-peroxidase-conjugated secondary antibody at a dilution of 1:2000 was reacted with the samples for 1 h, and then visualized with an ECL Western Blotting Detection System (Amersham Pharmacia Biotech Korea, Korea).

**Colorimetric Analysis of MMP Activity** The levels of MMP activity in the conditioned media were evaluated using an enzyme-linked immunosorbent assay (ELISA) kit (Biotrol Research Lab., Inc., PA, U.S.A.) according to the manufacturer’s instructions. The (Ac-Pro-Leu-Gly-2-mercapto-4-methyl-pentanoyl]-Leu-Gly-OC2H5) was used to evaluate the total MMP activity in the conditioned media. This substrate is efficiently cleaved by all MMPs tested so far. The (3-[2,4-dinitro-phenyl]-1-2,3-diaminopropionyl)-Pro-Tyr-Ala-Arg-NH2 (Bachem) was used to evaluate the human recombinant proenzyme MMP-3 (Oncogene, Darmstadt, Germany) activity, and (7-methoxyxocumin-4-y)-acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitro-phenyl]-1-2,3-diaminopropionyl)-Ala-Arg-NH2 (Bachem) was used to evaluate the human recombinant proenzyme MMP-13 activity. To assess the proteolytic activity, 25 μl of each sample was
pipetted into 96-well plates, together with diluted proMMP-3 and proMMP-13 enzyme, buffer, and substrate. After 1 h of incubation at 37 °C, the samples were measured at 405 nm. For each sample, MMP-3 and MMP-13 activities were measured as a percentage of the MMPs in that culture well.

Measurement of Lactate Dehydrogenase Activity As an indicator of cell viability, cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in the culture medium. An optimized LDH test (Promega Corp., Madison, WI, U.S.A.) was used to quantify LDH activity in the medium of the cartilage explant cultures.

Histological Analysis Cartilage explant pieces were fixed in 10% neutral formalin, dehydrated with graded ethanol, embedded in paraffin, and sectioned into 4 μm slices. Sectioned tissues were stained with hematoxylin and eosin (H&E) for light microscopic examination. To detect proteoglycan and collagen in the cartilage, duplicate sections were stained with Safranin O and Masson’s Trichrome. The number of chondrocytes was measured in three identically treated cartilage explants using a 200× lens. A pathologist with no prior knowledge of the test reagents examined the stained slides.

Statistical Analysis The results were expressed as means±S.D. calculated from the specified numbers of determinations. Statistically significant differences relative to the untreated control group were calculated by Student’s one-tailed paired t test. Differences with p values <0.05 were deemed statistically significant.

RESULTS

Dose Response and Time Course of IL-1α-Induced Cartilage Degradation In preliminary experiments, to optimize the conditions with which to induce proteoglycan and collagen degradation, rabbit articular cartilage was cultured with 1, 2.5, 5, 10, or 20 ng/ml IL-1α for 14 d. These effects were dose-dependent, and 5 ng/ml IL-1α was required to consistently achieve the maximal response. In experimental cultures of rabbit cartilage treated with 5 ng/ml IL-1α, more than 73% of GAG had been released from the tissue after 3 d of culture, and about 75% after 14 d (Fig. 1A). In parallel experiments, cartilage explants were cultured with various concentrations of IL-1α for 14 d. There was little release of type II collagen from the cartilage at any concentration of IL-1α for 3 d, after which there was a marked increase in collagen release to about 75% by day 14 of culture (Fig. 1B).

Effect of B. platyphilla on Proteoglycan and Collagen Degradation To study whether B. platyphilla affects proteoglycan and collagen degradation in rabbit cartilage explants, rabbit cartilage explants were cultured in the presence of 5 ng/ml IL-1α for 28 d. B. platyphilla consistently reduced the IL-1α-mediated GAG release into the culture medium until 14 d (Fig. 2). No release of collagen into the culture medium was observed from explants treated with IL-1α alone until 7 d. After 14 d, B. platyphilla markedly reduced collagen degradation in the explants relative to that in the IL-1α-treated cultures, and significantly reduced degradation until 28 d (Fig. 2). Moreover, B. platyphilla dose-dependently reduced IL-1α-mediated GAG and collagen release into the culture medium (Fig. 3). B. platyphilla significantly reduced GAG and collagen release starting from a low concentration of 0.02 mg/ml, and almost totally inhibited it at a concentration of 0.2 mg/ml for 14 d. For the vehicle, 0.1% DMSO did not affect GAG or collagen release compared with the non-treated DMSO control for culture periods. Rofecoxib, a selective cyclooxygenase (COX)-2 inhibitor, did not inhibit GAG or collagen degradation at a concentration of 30 μM. Diclofenac, a non-selective COX-2 inhibitor, effectively reduced GAG and collagen degradation at 30 μM (Fig. 3).

Effect of B. platyphilla on MMP Expression and Activities We examined whether B. platyphilla inhibited IL-1α-mediated MMP-3 and MMP-13 mRNA, proteins and their
activities in cartilage explant culture. *B. platyphylla* inhibited MMP-3 and MMP-13 mRNA synthesis at 14 d of culture in a dose-dependent manner (Fig. 4A). Also, Western blot analysis showed that *B. platyphylla* at 0.02—0.2 mg/ml down-regulated proMMP-3 and proMMP-13 expression in rabbit cartilage explant culture (Fig. 4B). We also tested the levels of MMP-3 and MMP-13 activity in the medium from cultures after 14 d with or without *B. platyphylla*. MMP-3 and MMP-13 levels decreased dose-dependently in the culture media with *B. platyphylla* at day 14 compared with the levels in IL-1α-treated cultures (Fig. 5). In *B. platyphylla*-treated cultures, the levels of MMP-3 and MMP-13 activity were reduced more than in cultures treated with only BB94, an MMP inhibitor (Fig. 5).

**Effect of *B. platyphylla* on the Viability of Cartilage Explants** We examined whether *B. platyphylla* affects chondrocyte viability in cartilage explant cultures. We were unable to detect any LDH activity in the incubation medium of cultures treated with the drug or with IL-1α alone, indicating that neither IL-1α nor *B. platyphylla* have cytotoxic effects on chondrocyte cartilage explants during 3, 7, or 14 d of culture (Fig. 6). As a vehicle, 0.1% DMSO did not affect cell viability.

**Effect of *B. platyphylla* on the Morphology of Cartilage Explants** We evaluated whether *B. platyphylla* affects the structural integrity of cartilage or chondrocytes in IL-1α-induced cartilage explant cultures. Examination of sections of untreated cartilage explants revealed normal staining for proteoglycan and collagen with Safranin O and Masson’s Trichrome (Fig. 7). In contrast, microscopic analysis of IL-1α-treated explants showed a reduction in the amounts of proteoglycan and collagen present. *B. platyphylla*-treated cartilage showed more intense staining for proteoglycan and collagen compared with cartilage samples treated with IL-1α alone (Fig. 7). The total number of chondrocytes increased...
ng/ml IL-1α tentatively achieves the maximal response of about 73% with 5 in the absence or presence of B. platyphylla. (A) Proteoglycan was determined by Safranin O staining. Arrow: chondrocytes of mitosis (Original magnification ×200). (B) Collagen expression was determined using Masson’s Trichrome staining.

2.7-fold after 14 d in culture, compared with the number in cartilage treated with IL-1α alone. Neither IL-1α alone nor additional treatment with B. platyphylla induced any pathological changes in the cartilage explants (Fig. 7).

**DISCUSSION**

Betula platyphylla var. japonica is an oriental medicinal herb used for the treatment of osteoarthritis. However, the current problem facing us is, “How can we exert a protective effect on cartilage?” Therefore, we investigated the effects of Betula platyphylla var. japonica on the release of proteoglycan, the degradation of collagen, and the mechanisms involved, in IL-1α-treated rabbit cartilage explants.

In general, the destruction of cartilage in OA is initially caused by a decrease in its proteoglycan content, followed by the degradation of collagen fibers. Some studies have suggested that investigation into cartilage degradation should include an examination of both proteoglycan and the collagen matrix. In a preliminary study, we confirmed that proteoglycan is dose-dependently degraded by IL-1α, and consistently achieves the maximal response of about 73% with 5 ng/ml IL-1α applied to rabbit explants for 14 d of culture (Fig. 1A).

Collagen degradation by IL-1α significantly increased to about 72.5% after 14 d (Fig. 1B). Under these conditions, cartilage explants cultured for 28 d still released about 79% of type II collagen into the culture medium (data not shown). Previous investigators have shown that IL-1α induced the degradation of more than 70% of proteoglycan after 3 d, and about 65% of collagen after 15—25 d in bovine explants and rabbit cartilage explants.

We investigated the protective effects of B. platyphylla on IL-1α-mediated proteoglycan and collagen release in rabbit cartilage explant cultures. In this study, B. platyphylla dose-dependently reduced IL-1α-mediated proteoglycan release into the culture medium between 3 and 14 d (Figs. 2, 3). The release of collagen was not observed in culture medium treated with IL-1α until 7 d. B. platyphylla markedly reduced collagen degradation after 14 d in a concentration-dependent manner compared with that in IL-1α-treated cultures (Figs. 2, 3). These results suggest that B. platyphylla is effective for the reduction of proteoglycan and collagen degradation in rabbit cartilage. The control, diclofenac, a non-selective COX-2 inhibitor, showed inhibitory effects, whereas rofecoxib, a selective COX-2 inhibitor, showed none (Fig. 3). These results are in agreement with those of others. Studies by Ito et al. have reported that diclofenac has a positive effect on the inhibition of cartilage metabolism.

B. platyphylla inhibited MMP-3 and MMP-13 mRNA and protein expression, as well as their activity, in articular cartilage explants (Figs. 4, 5). In our experiments, we demonstrated that B. platyphylla markedly and dose-dependently down regulated MMP-3 and MMP-13 expression (Fig. 4), and colorimetric analysis demonstrated that MMP-3 and MMP-13 activities were similarly inhibited when tested at extract concentrations of 0.02—0.2 mg/ml (Fig. 5). These results suggest that B. platyphylla is an effective inhibitor of cartilage loss. Proteoglycan is particularly vulnerable to proteinase attack and is therefore a sensitive indicator of proteolytic activity. Studies by Lin et al. have suggested that MMP-3 is the proteinase mainly responsible for the release of proteoglycan and collagen as fragments after cartilage resorption *in vitro* and *in vivo*, because they are produced by cleavage of the aggrecan molecule at the position cleaved by MMP-3. Furthermore, Kochzi et al. suggested that MMP-13 plays a role in the cartilage destruction stimulated by IL-1β, and breaks down type II collagen in bovine nasal cartilage explants.

We have also shown that B. platyphylla had no impact on the viability of cartilage explants, when determined on days 3, 7, and 14 of the culture period (Fig. 6). The distribution of proteoglycan and collagen determined using Safranin O and Masson’s Trichrome staining demonstrated the disorganization of the articular cartilage, including fibrillation, fissures, chondrocyte nuclear cleavage, and cluster formation in the B. platyphylla-treated group compared with the control (Fig. 7). Chondrocyte enlargement, resulting in giant chondrocyte containing multiple nuclei, was also observed in B. platyphylla-treated cartilage cultures (Fig. 7). Based on the above data regarding the metabolism, viability and morphology of cartilage, we suggest that B. platyphylla is potentially useful
in the treatment of degenerative joint disease.

However, further investigation is required into the mechanism of action of \textit{B. platyphylla} in exerting its chondroprotective effect via aggrecanase expression, and to develop an effective regimen for the treatment of osteoarthritis. In summary, \textit{B. platyphylla} has an inhibitory effect on the release of proteoglycan and collagen associated with the downregulation of MMP-3 and MMP-13 activities, without affecting the viability or morphology of IL-1\textalpha-induced rabbit articular cartilage explants. We suggest that \textit{B. platyphylla} could act as an agent for pharmacological intervention in cartilage loss in the progress of osteoarthritis.

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REFERENCES