Increased Accumulation of Superficial Zone Protein (SZP) in Articular Cartilage in Response to Bone Morphogenetic Protein-7 and Growth Factors

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ABSTRACT: The purpose of this study was to investigate the role of bone morphogenetic proteins (BMPs), such as BMP-7, growth factors, and cytokines, in the accumulation of superficial zone protein (SZP) in bovine articular cartilage. Calf superficial articular cartilage discs and chondrocytes were obtained for explant and monolayer culture systems, respectively. Dose- and time-dependent actions of BMP-7 on SZP accumulation were investigated in both explant and monolayer culture systems. In addition, actions of various morphogens and growth factors [BMP-2, BMP-4, fibroblast growth factor 2 (FGF-2), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), and transforming growth factor β (TGF-β1)], and cytokines [interleukin (IL)-1α, IL-1β, and tumor necrosis factor (TNF-α)] alone, and in combination with BMP-7, on SZP accumulation were investigated in monolayer culture systems. SZP accumulation was quantified in both the cartilage and the medium using SDS-PAGE and subsequent immunoblotting. In both explant and monolayer cultures, BMP-7 increased SZP accumulation in a dose- and time-dependent fashion (p < 0.05). Furthermore, SZP accumulation was significantly increased in monolayer cultures by FGF-2, IGF-1, PDGF, and TGF-β1 (p < 0.05). Both IL-1α and TNF-α significantly reduced SZP accumulation (p < 0.05). The inhibition of SZP accumulation by TNF-α was partially alleviated by concurrent treatment with BMP-7. The results of this investigation provide novel insights into the role of morphogens, especially BMP-7, growth factors, and cytokines in the accumulation of SZP in articular cartilage. This information has clinical implications because stimulation of SZP may ameliorate the pathology of joint function in arthritis. Furthermore, tissue engineering approaches to articular cartilage may depend on the optimal synthesis and assembly of SZP in the superficial zone to ensure functional tissue architecture.

INTRODUCTION

Articular cartilage consists of surface, middle, and deep layers, each with a unique cell architecture, biochemical composition, and mechanical properties. Superficial zone protein (SZP) is a proteoglycan that is synthesized and secreted selectively by chondrocytes of the superficial layer of the articular cartilage. Unlike the major cartilage proteoglycan aggrecan, SZP is not retained in the matrix, but mostly secreted into synovial fluid. A thin layer of SZP is present at the uppermost region of the articular surface, bound to macromolecules in the lamina splendens, where it functions as a boundary lubricant. SZP has also been localized in synovial membrane lining joint cavities, tendon, and meniscus. Radin and Swann first enunciated the role for a glycoprotein fraction in joint lubrication. This glycoprotein was isolated and named lubricin. Subsequent studies established the homology of lubricin to megakaryocyte stimulating factor (MSP), SZP, hemangiopoietin (HAPO), and proteoglycan 4 (PRG4). The discrete functional domains in SZP suggest multiple biological functions, such as growth.
promotion, cytoprotection, and lubrication. Based on biochemical composition and localization at the uppermost surface layer of articular cartilage, SZP is the key mediator in boundary lubrication, and thus plays an important role in the overall functional integrity of diarthrodial joints. Moreover, mutations of SZP gene, located in chromosome 1q25, have been linked to camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, an autosomal recessive disease characterized by synovial hyperplasia without evidence of inflammation where ineffective lubrication apparently results in premature joint wear. It has also been reported that SZP gene is differently expressed in the synovium of rheumatoid arthritis and osteoarthritis, implying a possible role in the pathogenesis of these diseases. A recent investigation reported that mice lacking the PRG4 (SZP) gene demonstrated abnormal protein deposits on the cartilage surface, disappearance of the underlying superficial zone, synovial hyperplasia, and precocious failure of joint function.

It is well established that morphogens and growth factors play an important role in cartilage homeostasis. Bone morphogenetic proteins (BMPs) are pleiotropic regulators of cartilage and bone differentiation cascade including chemotaxis of progenitor cells, mitosis of mesenchymal stem cells, and differentiation of cartilage and bone. It has been shown that BMPs induce new bone formation and cartilage formation in vitro and in vivo. BMP-7, also called human osteogenic protein-1 (OP-1), plays an important role in human and bovine cartilage homeostasis and repair. Multiple studies have shown that BMP-7 and other growth factors can synergistically promote increased survival and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Basic fibroblast growth factor (FGF-2), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), and transforming growth factor 1 (TGF-β1) have all been shown to be anabolic for cartilage and chondrocytes.

Recent evidence suggests that inhibition of the action of growth factors may contribute to disease processes in rheumatoid and osteoarthritis. Such inhibition may be mediated by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), or interleukin-1 (IL-1). These agents promote cartilage matrix degradation in part by enhancing the expression of matrix metalloproteinases (MMPs). Furthermore, there is evidence that IL-1 and TNF-α co-localize with MMPs in the superficial layer of arthritic cartilage, emphasizing the key role of this layer in the pathogenesis of arthritic diseases.

Considering the suggested and potential biological functions of SZP, it is imperative to investigate factors that can regulate the biosynthesis of this proteoglycan. A few studies have investigated the role of growth factors and cytokines in the expression of SZP in articular cartilage. SZP expression can either be up- or downregulated in response to treatment with TGF-β1 or IL-1α, respectively. However, no systematic study has examined the influence of BMPs on SZP accumulation in articular cartilage. Thus, our first objective was to study the role of BMP-7 on SZP accumulation in both explant and monolayer cell culture models of bovine superficial articular cartilage. The second objective was to investigate the interplay between BMP-7 with other growth factors and cytokines, such as BMP-2, BMP-4, FGF-2, IGF-1, PDGF, TGF-β1, IL-1α, IL-1β, and TNF-α, on SZP accumulation in monolayer cell cultures of bovine superficial articular chondrocytes.

**MATERIALS AND METHODS**

**Tissue Acquisition**

Stifle (knee) joints from 3-month-old calves were obtained within 6 h of slaughter from a local abattoir. The joints were opened under aseptic conditions, exposing the femoral condyles. In preliminary pilot experiments we observed varying SZP accumulation across different surface regions of lateral and medial femoral condyles. Therefore, in order to minimize possible variations in SZP surface distribution, cartilage tissue was only obtained from the central load-bearing region of the lateral femoral condyle of each joint (Fig. 1A).

For explant culture, two full-thickness osteochondral plugs were obtained using a 5-mm diameter cork borer (Fisher Scientific, Hampton, NH). To remove any adherent synovial fluid, the plugs were rinsed three or four times with phosphate-buffered saline (PBS). The superficial 300-μm layer of each plug was then separated using a custom-built slicing device (Fig. 1B). The resulting cartilage discs were divided into equal halves to obtain a total of four explants per knee joint.

For cell culture, a Silvers miniature skin graft knife (Integra, Plainsboro, NJ) was used to shave off the top 150 to 200 μm from the central region of the lateral femoral condyle. After rinsing the tissue slices with PBS containing antibiotic solution, superficial layer chondrocytes were isolated by digestion with 0.2% collagenase-P (Roche Pharmaceuticals, Nutley, NJ) for 5 h.

**Culture of Cartilage Explants and Chondrocytes**

Cartilage discs were plated at 1 disc per 1 mL medium per well (24-well plate) and equilibrated for 48 h in a
tissue incubator (37°C, 5% CO₂) with serum-free, chemically defined low glucose Dulbecco’s modified Eagle medium: nutrient mixture F12 (Ham) 1:1 (DMEM/F-12, Invitrogen, Carlsbad, CA), supplemented with 0.1% bovine serum albumin (cell culture grade, 96% purity), 100 units/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL ascorbate-2-phosphate. Medium was changed after the equilibration period and explants were treated according to the protocol described in the experimental design section.

Isolated chondrocytes in monolayer cultures were plated at a high density (400,000 cells/500 µL medium/well) in 24-well tissue plates. Cells were first equilibrated for a period of 24 h. For monolayer cultures, the culture medium (described previously) was additionally supplemented with 5% fetal bovine serum (FBS). Medium was changed after the equilibration period and cells were treated according to the experimental design described below.

Experimental Design

Explant and cell culture experiments were designed to investigate the role of BMPs, growth factors, and cytokines in the accumulation of SZP. Results are presented as means and standard deviations for each experiment (n = 6).

Dose-Dependent Action of BMP-7 on SZP Accumulation in Explant and Monolayer Cell Cultures

Explants from the same animal were assigned to an untreated control and graded levels (100, 300, 1000 ng/mL) of recombinant human BMP-7 (generously provided by Dr. T. K. Sampath, Creative Biomolecules, Hopkinton, MA) for a period of 3 days. The doses of BMP-7 utilized are based on previous work from our laboratory. At the end of the treatment interval the explants and culture media were flash frozen in liquid nitrogen for subsequent analysis. Similarly, monolayer cell culture wells from the same animal were assigned to a control and increasing concentrations of BMP-7. Culture media were collected at day 3 and cells were trypsinized with 0.25% trypsin-EDTA (Invitrogen) and recounted using a hemocytometer (American Optical, Buffalo, NY).

Time-Dependent Action of BMP-7 on SZP Accumulation in Explant and Monolayer Cell Cultures

Explants and cell cultures from the same animal were treated with 300 ng/mL BMP-7 for a period of 1, 3, or 7 days. An untreated control was assigned to each time group. For the 7-day group, half of the culture medium was changed on day 3 and replaced with fresh medium supplemented with 300 ng/mL of BMP-7. Tissue and media were collected as described above.

Actions of Growth Factors and Cytokines Alone and in Combination with BMP-7 on SZP Accumulation in Monolayer Cell Cultures

Monolayer cell cultures were treated with 100 ng/mL each of BMP-2, BMP-4, or BMP-7, and 30 ng/mL each of FGF-2, IGF-1, PDGF, or TGF-β1 (R & D Systems, Minneapolis, MN) for 3 days. To investigate potential
synergistic effects of BMP-7 with other growth factors, cell cultures were incubated with 100 ng/mL of BMP-7 in the presence or absence of 30 ng/mL of FGF-2, IGF-1, PDGF, or TGF-β1 for 3 days. In addition, cell cultures were incubated for 3 days with 10 ng/mL of IL-1α, IL-1β, or TNF-α (R & D Systems) in the presence or absence of 100 ng/mL of BMP-7. These doses are based on previous studies involving articular cartilage metabolism.44,46–48 After the treatment period media samples were collected for analysis.

Protein Extraction and SZP Quantification

Protein extraction from explants was accomplished using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY) in 1 mL of 4 M GuHCl containing 0.2% 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS), 10 mM EDTA, and 0.05 M Tris, pH 7.2 containing a protease inhibitor cocktail49 (Sigma-Aldrich, St. Louis, MO). Homogenized cartilage was frozen in liquid nitrogen and thawed for three cycles to ensure the release of proteins, followed by centrifugation at 14,000 g for 10 min. The supernatant (protein) was collected and buffer-exchanged in YM-10 Centricon filter devices (Millipore, Billerica, MA) with 6 M urea containing 0.05 M Tris (pH 7.4). The protein in the extract was quantified using Lowry assay.50

SDS-PAGE followed by immunoblotting was performed to compare the relative amount of SZP produced by untreated control and treated groups. For cartilage protein extracts, the loading volume was based on 10 μg/mL of total protein, as determined by the Lowry assay. The loading volume for media samples was 20 μL. The samples were added to the appropriate amount of reducing loading buffer, boiled for 5 min, and then electrophoresed on 5% SDS-polyacrylamide gels. To ensure that equal amounts of media were loaded, the bottom portion (below 75 kDa marker) of each polyacrylamide gel was cut, and additionally stained for BSA (66 kDa) using Coomassie blue stain. The remainder of the gel was then transferred to a PVDF membrane using trans-blotting device for 30 min at 25 V (BIO-RAD Laboratories, Hercules, CA). Membrane was blocked with 5% nonfat dried milk in TBS T (25 mM Tris–HCl, 125 mM NaCl, and 0.1% Tween 20) for 1 h and probed with monoclonal mouse SZP antibody MAb S6.79 (1 μg/mL) in 5% milk–TBS T (1:5000 dilution) overnight at 4°C.51 Detection was done with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies in 5% milk–TBS T (1:3000 dilution) for 1 h at room temperature. SZP in the media of explant cultures was further quantified using ELISA as described previously by Schmid and colleagues.51

Visualization of immunoreactive proteins was achieved using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL). This was performed by first mixing the two substrate components at a 1:1 ratio to prepare the substrate working solution. Each blot was then incubated for 1 min in SuperSignal substrate working solution. The excess reagent was drained and the blot was covered with a clear plastic wrap. The blot was then exposed to X-ray film. X-ray films were scanned and the amount of SZP was represented, using AlphaEase FC image analysis software (Alpha Innotech, San Leandro, CA), as relative units of band density to untreated controls.

Statistical Analysis

A one-way analysis of variance (ANOVA) was performed using StatView statistic (SAS Institute Inc., Cary, NC) to determine the effects of BMPs, growth factors, and cytokines on SZP accumulation. Statistically significant differences between groups were further investigated using Fisher’s PLSD (protected least significant difference) post-hoc test with the level of significance defined as p < 0.05.

RESULTS

Dose-Dependent Action of BMP-7 on SZP Accumulation in Articular Cartilage Explant Cultures

A comparison of the effects of the different doses of BMP-7 on SZP levels revealed dose-dependent responses in both cartilage and media from explant cultures (Fig. 2A). However, relative differences in SZP levels between untreated control and treatment groups were only statistically significant (p < 0.05) in the media samples (Fig. 2B). In addition, relative differences of SZP in the medium were significant among the 100/300 (p = 0.018), 100/1000 (p < 0.0001), and 300/1000 (p = 0.0022) treatment groups. There were no statistically significant differences in SZP content in the cartilage samples (Fig. 2B). Furthermore, the amount of SZP that accumulated in the culture media was significantly higher than that present in the explants after harvest. The quantification of SZP levels in the media of explants was confirmed by enzyme-linked immunosorbent assay (ELISA; Fig. 2C).

Time-Dependent Action of BMP-7 on SZP Accumulation in Articular Cartilage Explant Cultures

Similar trends in SZP accumulation were observed when explant cultures were incubated for 1, 3, or 7 days with 300 ng/mL of BMP-7. Significant differences in SZP levels were only present between the untreated control and the treatment groups in media samples (Fig. 3A,B). Compared to their respective controls, the amount of SZP in culture medium was significantly higher for day 3 (p = 0.005), and day 7 (p = 0.0001), but not day 1 (p = 0.2538) treatment groups (Fig. 3).
Figure 2. Dose-dependent action of BMP-7 on SZP accumulation in superficial explant cultures: (A) Immunoblots of cartilage and media samples from explant cultures incubated in the absence (C) or presence of graded levels (100, 300, 1000 ng/mL) of BMP-7 for 3 days. (B) The amount of the SZP protein from image analysis of immunoblots (n = 6) represented as relative units of band density compared to untreated controls. A value of 100 was arbitrary assigned to cartilage control samples. (*) indicates a statistically significant difference (p < 0.05). Significant differences also existed between the different BMP-7 treated samples in media (not shown). (C) SZP accumulation (μg/mL) in the media of explants in response to graded levels of BMP-7 was further quantified by ELISA.
Like the previous experiment, SZP that accumulated in the culture media was significantly higher than that present in the explants after harvest.

**Dose- and Time-Dependent Actions of BMP-7 on SZP Accumulation in Articular Cartilage Monolayer Cell Cultures**

Similar patterns of SZP accumulation were observed in both dose- and time-dependent treatments with BMP-7. Compared to untreated controls, cells treated with 100, 300, and 1000 ng/mL BMP-7 for 3 days synthesized 18.1% ($p = 0.0477$), 44.2% ($p < 0.0001$), and 71.6% ($p < 0.0001$) more SZP, respectively (Fig. 4B). Cell cultures incubated for 1 day with 300 ng/mL of BMP-7 accumulated only 18.9% ($p = 0.2182$) more SZP, while those incubated for 3 and 7 days produced 28% ($p = 0.0012$) and 17.1% ($p = 0.0046$) more SZP (Fig. 4A). Additionally, the results were controlled for cell number because the average cell numbers between untreated controls and treatment groups at the end of each time interval were not significantly different ($p > 0.05$).

**Actions of Growth Factors and Cytokines Alone and in Combination with BMP-7 on SZP Accumulation in Articular Cartilage Monolayer Cell Cultures**

Compared to untreated controls, chondrocytes treated with FGF-2, IGF-1, PDGF, and TGF-$\beta$1 produced significantly more SZP ($p < 0.05$), while those incubated with BMP-2 ($p = 0.3198$), and BMP-4 ($p = 0.9192$) did not (Fig. 5). Furthermore, compared to BMP-7 treated cells, only chondrocytes treated with IGF-1 ($p = 0.002$) and TGF-$\beta$1 ($p < 0.0001$) accumulated significantly more SZP. The effect of growth factor combination on SZP accumulation was also investigated. Addition of BMP-7 to FGF-2, IGF-1, PDGF, or TGF-$\beta$1 did not yield significantly more SZP than treatment with either growth factor alone.

Compared to untreated controls, treatment with IL-1$\alpha$, IL-1$\beta$, and TNF-$\alpha$ resulted in downregulation of SZP levels (Fig. 6). However, only IL-1$\alpha$ and TNF-$\alpha$ showed significant decreases of 30.5% ($p = 0.012$) and 23.5% ($p = 0.008$), respectively. Moreover, compared to the isolated effect of each cytokine, BMP-7 resulted only in a significant SZP increase when added to TNF-$\alpha$ (27.5%; $p = 0.015$).
Despite this increase, SZP expression in TNF-α treated cells was still below control. Similar to BMP-7 experiments above, all the results were controlled for cell number.

**DISCUSSION**

The aim of this investigation was to determine the role of morphogens such as BMPs, growth factors, and cytokines such as FGF-2, IGF-1, PDGF, TGF-β1, IL-1α, IL-1β, and TNF-α in the accumulation of articular cartilage superficial zone protein, or SZP. Using SDS-PAGE followed by immunoblotting, we compared differences in band integrated densities between control and treatment groups, relatively quantifying the amount of SZP in explant and monolayer cell culture systems of chondrocytes from superficial layer of bovine articular cartilage.

In both explant and monolayer culture systems, BMP-7 increased SZP accumulation in a dose- and time-dependent fashion. The amount of SZP expressed in the culture medium was significantly higher than the amount present in the cartilage (Figs. 2 and 3). This finding is consistent with that fact that the majority of this proteoglycan is secreted into the synovial fluid. Moreover, SZP released into the culture medium was predominantly synthesized by the superficial layer chondrocytes, rather than SZP released from the articular surface because the amount of SZP in the media greatly exceeded the amount of SZP present in the cartilage at the end of each incubation period. Schmidt and colleagues reported that PRG4 (SZP) secretion into the medium varied with culture duration in the various culture systems, decreasing with time over a period of 7 to 9 days in the explant culture, and increasing with time in monolayer culture. In contrast, we found that SZP accumulation in the culture medium increased with culture duration in both explant and monolayer systems for up to 7 days. It is likely that the rate of SZP secretion into the culture medium might depend on a “concentration gradient” between the tissue and the medium, which might regulate SZP synthesis and secretion based on a potential feedback mechanism. Clearly, additional studies need to be conducted to investigate whether or not such a relationship exists.

We found that SZP levels did not significantly increase in the BMP-7 treated cartilage samples when compared to untreated controls (Figs. 2 and 3). A possible explanation is that because the majority of the synthesized SZP is secreted, significant differences in SZP accumulation between control and treatment groups could only be detected in the culture media. This was confirmed with ELISA (Fig. 2C). Another potential explanation for this disparity is based on the possibility that SZP content may vary in the surface regions, as suggested by other investigators. Given the nature of SZP, acting as a boundary lubricant, it is possible that its concentration across
the femoral condyles correlates with areas of contact pressure, being highest in regions with the highest pressures and vice versa. This potential surface variability could have accounted for the relatively large standard deviations seen for the cartilage samples in our study. Future studies are needed to evaluate the distribution of SZP across contact surfaces within the joint and to elucidate the role of mechanical stimulation in its synthesis, distribution, and secretion.

SZP accumulation also varied with different growth factor treatments in monolayer cell cultures. In addition to being upregulated by BMP-7, SZP levels also significantly increased when cells were treated with FGF-2, IGF-1, PDGF, or TGF-β1, but not BMP-2 or -4. It is known that BMPs are potent inducers of bone/cartilage differentiation and formation in vitro and in vivo. BMP-2, -4, and -7 are all expressed during joint development. BMP-2 appears to be a more potent stimulator of proteoglycan and collagen II synthesis in human mesenchymal stem cells than BMP-4. A study by Anderson and colleagues found that rat articular chondrocytes from the surface zone positively stained for BMP-7, but not BMP-2, or -4. Furthermore, it has been shown that human articular chondrocytes at different ages and histopathological stages are able to synthesize BMP-7. More specifically, the active form of BMP-7 is expressed primarily in the superficial layer of newborn and adult human articular cartilage, whereas it is expressed in all the zones of osteoarthritic cartilage. The fact that BMP-7, as compared to BMP-2 or -4, was a more potent stimulator in PRG4 synthesis, could relate to its selective presence and function as a homeostatic morphogen, critical for the integrity of the superficial zone chondrocytes in the articular cartilage.

SZP accumulation was also enhanced by FGF-2, IGF-1, PDGF, and TGF-β1, with TGF-β1 having the strongest response (Fig. 5). This is consistent with previous studies showing that TGF-β1 is a
potent stimulator of SZP synthesis. In contrast to previous reports showing synergism among different growth factors in cartilage and bone, the addition of BMP-7 to both IGF-1 and TGF-β1 did not increase the amount of SZP produced by the chondrocytes. Catabolic cytokines, such as IL-1α, IL-1β, and TNF-α, suppressed SZP levels in monolayer cell cultures. This finding is consistent with other studies on articular cartilage SZP. It is noteworthy that the inhibitory effect of TNF-α was partially reversed by the addition of BMP-7 in the present investigation. However, on the other hand, BMP-7 was unable to reverse the inhibitory effects of IL-1α and IL-1β.

In conclusion, our results provide novel insights into the role of morphogens, especially BMP-7, growth factors, and cytokines in the accumulation of SZP in articular cartilage. This information has clinical implications because stimulation of SZP by morphogens and growth factors may slow the progression of pathological conditions such as rheumatoid arthritis and osteoarthritis. Furthermore, tissue engineering approaches to articular cartilage for restoration of joint function may depend on the optimal synthesis and assembly of SZP in the surface of the articular cartilage.

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REFERENCES


