Modulation of Superficial Zone Protein/Lubricin/PRG4 by Kartogenin and Transforming Growth Factor- β 1 in Surface Zone Chondrocytes in Bovine Articular Cartilage

Cartilage
2016, Vol. 7(4) 388–397
© The Author(s) 2016
Reprints and permissions:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1947603516630789
cart.sagepub.com

Kazumasa Miyatake¹, Kenjiro Iwasa¹, Sean M. McNary¹, Gordon Peng¹, and A. Hari Reddi¹

Abstract

Objective. Superficial zone protein (SZP)/lubricin/PRG4 functions as a boundary lubricant in articular cartilage to decrease friction and wear. As articular cartilage lubrication is critical for normal joint function, the accumulation of SZP at the surface of cartilage is important for joint homeostasis. Recently, a heterocyclic compound called kartogenin (KGN) was found to induce chondrogenic differentiation and enhance mRNA expression of lubricin. The objective of this study was to determine whether KGN can stimulate synthesis of SZP in superficial zone, articular chondrocytes. **Design.** We investigated the effects of KGN and transforming growth factor- β 1 (TGF- β 1) on articular cartilage and synovium of the bovine knee joint by evaluating SZP secretion by enzyme-linked immunosorbent assay analysis. Monolayer, micromass, and explant cultures of articular cartilage, and monolayer culture of synoviocytes, were treated with KGN. SZP accumulation in the medium was evaluated and mRNA expression was measured through quantitative polymerase chain reaction. **Results.** TGF- β 1 stimulated SZP secretion by superficial zone chondrocytes in monolayer, explant, and micromass cultures as expected. In addition, SZP secretion was inhibited by IL-1 β in explant cultures, and enhanced by TGF- β 1 in synoviocyte monolayer cultures. Although KGN elicited a 1.2-fold increase in SZP mRNA expression in combination with TGF- β 1, KGN neither stimulated any significant increases in SZP synthesis nor prevented catabolic decreases in SZP production from IL-1 β . **Conclusions.** These data suggest that the chondrogenic effects of KGN depend on cellular phenotype and differentiation status, as KGN did not alter SZP synthesis in differentiated, superficial zone articular chondrocytes.

Keywords

superficial zone protein (SZP), lubricin, kartogenin, transforming growth factor- β 1 (TGF- β 1), articular cartilage

Introduction

Osteoarthritis (OA) is a disease involving degradation of articular cartilage with attendant pain and impaired mobility. It is reported that OA affects more than 27 million people in the United States (2005) and it is estimated that by the age of 65 years, 80% of the U.S. population will exhibit radiographic evidence of OA.¹ Normal articular cartilage on the ends of long bones in diarthrodial joints maintains a well-lubricated surface with an extremely low coefficient of friction for optimal joint mobility.² Weight bearing of loads up to 18 MPa is accomplished through an extracellular matrix architecture that combines the compressive properties of hydrated glycosaminoglycans (GAG) with the tensile strength of crosslinked type II collagen fibrils.³ Since articular cartilage is an avascular tissue with limited, innate potential for repair and regeneration,⁴ several therapeutic

strategies such as autologous chondrocyte implantation, mosaicplasty, and microfracture are used to repair articular cartilage. However, these treatments fail to regenerate tissue that resembles native articular cartilage, with zonal organization and functional lubrication.⁵ Articular cartilage is an anisotropic structure with a zonal design and consists of surface or superficial, middle, and deep zones. Superficial zone protein (SZP), also known as lubricin or proteoglycan

¹Department of Orthopaedic Surgery, Lawrence Ellison Center for Tissue Regeneration and Repair, School of Medicine, University of California, Davis, Sacramento, CA, USA

Corresponding Author:

A. Hari Reddi, Department of Orthopaedic Surgery, School of Medicine, University of California, Davis, Research Building I, Room 2000, Sacramento, CA 95817, USA.
Email: ahreddi@ucdavis.edu

4 (PRG4), is an ~345 kDa glycoprotein that is secreted into synovial fluid by surface zone chondrocytes and synovio-cytes.^{6,7} SZP localized in the superficial zone of articular cartilage reduces the coefficient of friction and wear in articular cartilage through a sacrificial, boundary lubrication mechanism.⁸⁻¹⁰ Moreover, mutation of the SZP gene, *prg4*, has been linked to camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, an autosomal recessive disease characterized by synovial hyperplasia, fouling of the articular surface, and precocious joint failure.¹¹ Mice lacking functional copies of the *prg4* gene also demonstrated precocious failure of joint function,¹² leading to OA.¹³ Therefore, SZP plays an important role in cartilage homeostasis. Given that superficial zone chondrocytes and synovium are the predominant sources of SZP in the synovial joint, we previously evaluated SZP production by protein analysis in various culture systems of cartilage and synovium, such as monolayer,^{7,14,15} micromass,¹⁶ explant,¹⁷⁻¹⁹ and pellet cultures.²⁰

Recently, Johnson *et al.*²¹ reported the discovery of a heterocyclic compound, kartogenin (KGN), from 22,000 structurally diverse, drug-like molecules using a high-throughput screening system. This small molecule binds to the carboxyl end of filamin A and disrupts its interaction with the transcription factor, core-binding factor β subunit (CBF β), and induces chondrogenic differentiation by regulating the CBF β -RUNX1 transcriptional program.²¹ KGN has been observed to enhance the expression of lubricin, aggrecan, and type II collagen in human mesenchymal stem cells (MSCs), and to have a chondroprotective, anti-catabolic effect in both *in vivo* and *in vitro* studies.²²⁻²⁴

It is well established that morphogens, such as bone morphogenetic protein-7 (BMP-7) and transforming growth factor- β 1 (TGF- β 1), and cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), play important roles in regulating SZP production.^{15,17,25} For example, TGF- β 1 stimulates SZP production while IL-1 β inhibits it. As SZP is secreted extracellularly into the synovial fluid *in vivo*, SZP synthesis is commonly measured by accumulation in the culture media *in vitro*.⁶ Motivated by previously published reports of KGN, the first aim of this investigation was to investigate the effect of KGN on SZP secretion in different tissue culture platforms such as monolayer, micromass, and cartilage explant cultures. The second objective was to investigate any potential interactions between KGN and the aforementioned regulatory cytokines, TGF- β 1 and IL-1 β , in these culture systems.

Methods

Tissue Acquisition

Stifle (knee) joints from 3-month-old calves were obtained from Research 87 (Boston, MA). The joints were dissected

under aseptic conditions, exposing the femoral condyles and the suprapatellar pouch.

Cell Isolation for Monolayer and Micromass Culture

Surface zone, bovine articular chondrocytes were obtained as described previously.¹⁵ Briefly, the surface zone of the articular cartilage was harvested from the anterior half of the lateral and medial femoral condyles (~100 μ m thick) using a dermatome. The articular cartilage from 6 to 8 bovine stifle joints from different animals were pooled. Then, osteochondral plugs were removed from the same femoral condyles using a coring reamer (Acuderm). Middle zone cartilage (1.25 mm slices) was removed from each plug using a custom, cutting jig. The synovium was harvested from the suprapatellar pouch. The cartilage slices and synovium were divided into small pieces with a razor blade, and digested with 0.2% collagenase P (Roche) in Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco, Carlsbad, CA, USA) along with a nutrient mixture of 50 μ g/mL ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), and antibiotics (Medium-A) with 3% fetal bovine serum (FBS; Gibco) for 2 hours at 37°C. Cells released from the tissues were filtered through a cell strainer (70 μ m; BD Biosciences, San Jose, CA, USA) and rinsed with DMEM/F12. The isolated chondrocytes or synovium-derived cells (synoviocytes) were plated in a monolayer at a density of 1×10^5 cells/well, in 12-well culture plates (Corning, NY, USA) in Medium-A with 10% FBS, and incubated at 37°C in a humid atmosphere of 5% CO₂ and 95% air. Following 24-hour equilibration in the culture medium, the medium was changed to fresh Medium-A with 1% ITS+ Premix (insulin-transferrin-selenium + linoleic acid; BD Biosciences) containing various concentrations of KGN (Tocris, Minneapolis, MN, USA) in the presence and absence of 3 ng/mL TGF- β 1 (R&D Systems, Minneapolis, MN, USA). All control cell cultures received an equal volume of the equivalent vehicle solution at the time of treatment. Cells were cultured for periods of 4 and 7 days. The culture media were collected and stored at -80°C for later analysis of SZP accumulation.

Micromass Culture

Isolated chondrocytes were suspended at a high density of 1×10^5 cells/20 μ L in Medium-A with 10% FBS, and incubated at 37°C in a humid atmosphere of 5% CO₂ and 95% air.^{16,26} A 20 μ L drop of cell suspension was gently placed in the center of each well of a 12-well plate. After waiting for 90 minutes, 1 mL of Medium-A with 10% FBS was slowly added to each well.^{16,26} Following overnight equilibration, the medium was changed to fresh Medium-A with 1% ITS+

Premix in the presence and absence of 3 ng/mL TGF- β 1 and 10 μ M KGN. The culture media were collected and stored at -80°C for later analysis of SZP accumulation. Cultures were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (Sigma Aldrich), in PBS for 3 hours. After rinsing in 3% glacial acetic acid (pH 1) 3 times, cultures were stained overnight in 0.5% Alcian blue (Sigma-Aldrich) in 0.1 N HCl (pH 1), and then destained in 3% glacial acetic acid.

Tissue Acquisition for Cartilage Explant Culture

Matched pairs were obtained from 8 different bovine stifle joints for cartilage explant cultures. Four osteochondral plugs were obtained in close proximity, using a 5 mm diameter coring reamer. Because of the limited size of juvenile bovine stifle joints, cartilage explants were obtained and utilized as a group from either the anterior region of the medial femoral condyle or trochlear groove, in order to conserve the number of joints employed. Comparable levels of SZP synthesis from these 2 locations have been reported.²⁷ From these 5 mm diameter cartilage disks, 2 mm diameter disks from the surface were obtained using an adjustable, custom-made jig. Explants were randomly assigned to control or treatment groups, pairwise. Cartilage disks (2 mm diameter) were submersed and equilibrated at 1 disk per 2 mL medium per well in 12-well culture plates in Medium-A with 1% ITS+ Premix, and incubated at 37°C in a moist atmosphere of 5% carbon dioxide and 95% air. The medium was replaced after a 24 hour equilibration period. Subsequently, the explants were switched to fresh Medium-A with 1% ITS+ Premix. Explants were pretreated with or without 10 μ M KGN for 2 hours before the addition of 10 ng/mL TGF- β 1 or 10 ng/mL IL-1 β (R&D Systems). Explants obtained from the medial femoral condyle were used for TGF- β 1 treatment, while explants obtained from the trochlear groove were used for IL-1 β treatment. All non-treated explant cultures received an equal volume of the equivalent vehicle solution at the time of treatment. Explants were cultured for 72 hours and the culture media were collected and stored at -80°C for later analysis of SZP accumulation.

Kartogenin Preparation

All KGN used in this study was obtained from a single lot, and was dissolved in dimethyl sulfoxide at a concentration of 10 mM. Recombinant, human TGF- β 1 was reconstituted in a filter sterilized solution of 0.1% BSA in 5 mM hydrochloric acid at 100 μ g/mL. Recombinant human IL-1 β was reconstituted in a filter sterilized solution of 0.1% BSA in PBS at 10 μ g/mL. Stock concentrations of all reagents were prepared and stored at -20°C , until they were diluted in culture media to their final concentrations.

Enzyme-Linked Immunosorbent Assay for SZP

Since the majority of SZP is secreted into the culture medium,⁵ the media was collected after treatment and SZP secretion was measured by an enzyme-linked immunosorbent assay (ELISA) with purified, bovine SZP as a standard.⁷ Briefly, each well of 96-well MaxiSorp plates (Nalge Nunc; Penfield, NY, USA) was coated with 1 μ g/mL peanut lectin (EY Laboratories; San Mateo, CA, USA) in 50 mM sodium carbonate buffer (pH 9.5). The wells were then blocked with 1% BSA in the same buffer for at least 2 hours. Aliquots of culture medium were incubated in the wells. Thereafter, the wells were incubated overnight with mAb S6.79 (1:5,000; a generous gift from Dr. T. Schmid, Rush Medical College, Chicago, IL, USA) as the primary antibody at 4°C ,²⁸ and then incubated at room temperature for 1 hour with goat anti-mouse IgG conjugated with horseradish peroxidase (1:3000; Bio-Rad; Hercules, CA, USA) as the secondary antibody. SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific; Waltham, MA, USA) was added, and the results were quantified in a luminometer. The wells were washed with PBS containing 0.05% Tween 20 after each step. SZP concentrations were calculated using a bovine SZP standard, which was purified by affinity chromatography on a peanut lectin column. Purity was verified by immunoblot analysis. The concentration of the SZP standard was quantified using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific).^{7,14}

Immunoblot Analysis

For immunoblot analysis, equal quantities of harvested culture medium were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfat dry milk in TBST (25 mM Tris HCl, 125 mM NaCl, and 0.1% Tween 20) for 1 hour and incubated overnight at 4°C with the primary antibody mAb S6.79 at a dilution of 1:5,000. The membranes were then incubated for 1 hour at 4°C with a horse radish peroxidase–conjugated secondary antibody at a dilution of 1:3,000 (Bio-Rad), followed by a 1 minute incubation with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) for visualization.

Quantitative Polymerase Chain Reaction Analysis

Messenger RNA (mRNA) expression levels were measured by quantitative polymerase chain reaction (q-PCR) according to the manufacturers' protocols. Briefly, total RNA was isolated from monolayer cell cultures using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Genomic DNA was removed through an on-column DNase I (Qiagen) digestion. Complementary DNA (cDNA) was reverse transcribed from mRNA using the SuperScript First Strand Synthesis System

(Invitrogen, Carlsbad, CA, USA). q-PCR was utilized for relative quantification of the cDNA using SYBR Green PCR Master Mix on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems; Foster City, CA, USA). SZP (*prg4*), collagen II (*col2a1*), aggrecan (*acan*), and Sox9 (*sox9*) expression levels (C_t) were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ΔC_t), computed relative to the untreated (i.e., control) group ($\Delta\Delta C_t$), and presented in terms of fold change ($2^{-\Delta\Delta C_t}$).²⁹ Primers for bovine SZP (Forward: 5'-AGAAAACCCGATGGCTATGA-3'; Reverse: 5'-TCGCCATCAGTCTAAGGAC-3'), collagen II (Forward: 5'-GCATTGCCTACCTGGACGAA-3'; Reverse: 5'-CGTTGGAGCCCTGGATGA-3'), aggrecan (Forward: 5'-GCGGGTGC GG GTCAA-3'; Reverse: 5'-TAGAATCCCGAGTCATTGGA-3'), Sox9 (Forward: 5'-ACGCCGAGCTCAGCAAGA-3'; Reverse: 5'-CAGAACGGCCGCTTCT-3'), and GAPDH (Forward: 5'-GGCGCCAAGAGGGTCAT-3'; Reverse: 5'-GTGGTTCACGCCCATCACA-3') were designed through Primer Express (Applied Biosystems) to span at least 1 intron and prevent nonspecific fluorescence arising from contaminating genomic DNA.^{14,30}

Statistical Analysis

For all monolayer and micromass cultures ($n = 6$), a 1-way analysis of variance (ANOVA) was used. Protein measurements were evaluated by the Tukey-Kramer honestly significant difference *post hoc* test. Groups not connected by the same letter were determined to be significantly different. Gene expression levels were assessed using the Dunnett's method *post hoc* test; groups significantly different than the untreated control were designated with an asterisk (*). Articular cartilage explant cultures ($n = 8$) were evaluated using a 2-way ANOVA to assess each treatment group as a factor, and utilized the Student *t* test as a *post hoc* test (2 treatment levels per factor). *P*-values less than 0.05 were considered statistically significant. All statistical analyses were performed using JMP 12 (SAS Institute, Cary, NC, USA). Data are presented as the mean $\pm$ standard deviation (SD).

Results

The Effects of KGN and TGF- β 1 on the Cellular Morphology of Superficial and Middle Zone Articular Chondrocytes

After 4 days of monolayer culture, untreated superficial zone chondrocytes were flattened and acquired a spread, fibroblastic morphology (Fig. 1A). On the other hand, the vast majority of middle zone chondrocytes maintained a round cell shape (Fig. 1B). Compared with the No Growth Factor group, TGF- β 1 treatment promoted increased cell

spreading in superficial zone chondrocytes; whereas no differences were observed in middle zone chondrocytes with TGF- β 1 treatment. No morphological alterations from treatment with KGN (10 nM, 100 nM, 1 μ M) were observed during culture in both superficial and middle zone chondrocytes.

The Effects of KGN and TGF- β 1 on SZP Accumulation and Gene Expression in Monolayer Culture

TGF- β 1 treatment enhanced SZP secretion significantly ($P < 0.0002$) compared with No Growth Factor groups in monolayer cultures of superficial zone chondrocytes (Fig. 2A). KGN stimulated SZP accumulation neither in the No Growth Factor groups (0 nM KGN, 0.8 ± 0.3 ; 10 nM KGN, 0.8 ± 0.2 ; 100 nM KGN, 0.9 ± 0.3 ; and 1 μ M KGN, 0.9 ± 0.3 μ g/mL; $P > 0.99$) nor in TGF- β 1-treated groups (0 nM KGN, 6.1 ± 1.9 ; 10 nM KGN, 4.8 ± 1.6 ; 100 nM KGN, 4.9 ± 1.6 ; and 1 μ M KGN, 4.4 ± 1.2 μ g/mL; $P > 0.24$), at all examined concentrations. In monolayer cultures of middle zone articular chondrocytes, no SZP was detected in the media as measured by immunoblot analysis (data not shown).

SZP mRNA expression was significantly upregulated ($P < 0.05$) 1.2-fold compared with non-treated control (0 μ M KGN, No Growth Factor) by a combination of TGF- β 1 and KGN, at KGN concentrations of 100 nM and 1 μ M (Fig. 2B). Whereas TGF- β 1-treated chondrocyte cultures experienced an upregulation of collagen II mRNA of over 2.7-fold relative to nontreated control (Fig. 2C), KGN appeared to elicit no response in collagen II expression. While mRNA expression of aggrecan (Fig. 2D) increased in response to 10 nM KGN in the presence of TGF- β 1 ($P < 0.0004$), all other treatment combinations did not result in substantial changes ($P > 0.19$). Sox9 gene expression did not change ($P > 0.08$) in response to any of the applied treatments (Fig. 2E).

The Effects of KGN and TGF- β 1 on SZP Accumulation in Micromass Culture

Alcian blue staining demonstrated that GAG production was enhanced in superficial (Fig. 3A) and middle (Fig. 3B) zone micromass cultures treated with the positive control treatment TGF- β 1 (TGF- β 1 and KGN + TGF- β 1), irrespective of the presence of KGN. In addition, all middle zone micromass cultures displayed stronger GAG staining than their respective treatment groups in the superficial zone micromass cultures. KGN had no apparent effect on GAG accumulation in the presence or absence of TGF- β 1. As in monolayer cultures, SZP secretion from superficial zone micromass cultures was increased by TGF- β 1, regardless of KGN treatment. While a faint immunoblot band was observed in the No Growth Factor (No GF; i.e., control) treatment group of

Figure 1. The effects of kartogenin (KGN) and transforming growth factor- β 1 (TGF- β 1) on the cellular morphology of superficial and middle zone articular chondrocytes. Primary, bovine, superficial and middle zone articular chondrocytes were cultured in monolayer for 4 days in serum-free, defined media supplemented with KGN (0 nM, 10 nM, 100 nM, and 1 μ M) and TGF- β 1 (0 and 3 ng/mL). **(A)** By day 4 of culture, the majority of superficial zone chondrocytes had attained a fibroblastic morphology. In comparison to the No Growth Factor group, TGF- β 1 treatment promoted increased cell spreading. **(B)** Middle zone chondrocytes maintained a round cell shape in both No Growth Factor and TGF- β 1 treatment groups. In both superficial and middle zone chondrocytes, all concentration of KGN elicited no apparent morphological alterations during culture.

superficial zone micromass cultures (**Fig. 3A**), no SZP was detected in immunoblots of middle zone micromass cultures (**Fig. 3B**). As was the case with GAG accumulation, KGN elicited no changes in SZP synthesis in superficial zone micromass cultures.

The Effects of KGN and TGF- β 1/IL-1 β on SZP Accumulation in Explant Culture

SZP is known to be modulated by morphogens and cytokines.^{7,15,31,32} This abundant evidence strongly suggests that the stimulatory effects of TGF- β 1 may be used as a positive control in experimental studies, as well as IL-1 β as a negative control because of its inhibitory effects. Thus, cartilage explants were also treated with TGF- β 1 (**Fig. 4A**) and IL-1 β (**Fig. 4B**) in order to determine whether there were any interactions between KGN and these cytokines. A 2-way

ANOVA revealed no significant interactions between KGN and neither TGF- β 1 ($P > 0.85$) nor IL-1 β ($P > 0.83$). The TGF- β 1 treatment level was significantly greater ($P < 0.017$) than the No Cytokine treatment level (**Fig. 4A**). Similarly, the IL-1 β treatment level was significantly lower ($P < 0.05$) than its respective No Cytokine level (**Fig. 4B**). In both cases, KGN had no effect ($P > 0.12$) as the 10 μ M KGN level was not statistically different than the 0 μ M KGN level.

The Effects of KGN and TGF- β 1 on Synovial Cells in Monolayer Culture

In monolayer culture, synovial cells showed fibroblastic morphology regardless of treatment (data not shown). Although TGF- β 1 treatment (0 μ M KGN, 0.37 ± 0.16 ; 10 μ M KGN, 0.32 ± 0.17 μ g/mL) enhanced SZP

Figure 2. The effects of kartogenin (KGN) and transforming growth factor- β 1 (TGF- β 1) on superficial zone protein (SZP) media accumulation of superficial zone articular chondrocytes. **(A)** Primary, superficial zone articular chondrocytes were treated with 0 nM, 10 nM, 100 nM, and 1 μ M of KGN in the presence and absence of 3 ng/mL TGF- β 1. SZP secretion was quantified by enzyme-linked immunosorbent assay, and validated by immunoblot as shown. TGF- β 1 enhanced SZP secretion as expected ($P < 0.0002$). At all applied doses, KGN appeared to have no effect on SZP synthesis, in either the presence ($P > 0.24$) or absence ($P > 0.99$) of TGF- β 1. Groups not connected by the same letter were determined to be significantly different. At the conclusion of the 4-day culture, mRNA expression was assayed via quantitative polymerase chain reaction (q-PCR). **(B)** KGN in concentrations of 100 nM and 1 μ M significantly increased SZP expression 1.2-fold in the presence of TGF- β 1 ($P < 0.05$). **(C)** While mRNA expression of collagen II was significantly increased by TGF- β 1 ($P < 0.0001$), **(D)** aggrecan expression was only enhanced by 10 nM KGN in the presence of TGF- β 1 ($P < 0.0003$). **(E)** Sox9 expression was unaffected by any of the treatments employed ($P > 0.0885$). An asterisk (*) indicates a statistically significant difference between that treatment group and untreated control. Values are presented as mean $\pm$ SD ($n = 6$).

Figure 3. The effects of kartogenin (KGN) and transforming growth factor- β 1 (TGF- β 1) on superficial zone protein (SZP) secretion from superficial and middle zone articular chondrocytes in micromass culture. Primary, bovine, superficial (A) and middle (B) zone articular chondrocytes were cultured as micromass pellets for 14 days in serum-free, defined media supplemented with and without KGN (10 μ M) and TGF- β 1 (3 ng/mL). Compared with No Growth Factor (No GF) and KGN treated groups, alcian blue staining revealed increased production of glycosaminoglycans (GAG) in superficial zone (A) and middle zone (B) micromass pellets treated with TGF- β 1. KGN did not appear to have an effect on GAG synthesis in either superficial or middle zone cultures. As shown by the representative SZP immunoblots, SZP secretion was increased by TGF- β 1 treatment in superficial zone chondrocyte micromass cultures (A). No SZP was detected in the media of middle zone chondrocyte micromass cultures (B). KGN treatment had a negligible effect on SZP secretion in micromass cultures.

Figure 4. The effects of kartogenin (KGN), transforming growth factor- β 1 (TGF- β 1), and interleukin-1 β (IL-1 β) on superficial zone protein (SZP) secreted from cartilage explants. Bovine articular cartilage explants were treated with and without KGN (10 μ M) in the presence and absence of TGF- β 1 (10 ng/mL) (A) or IL-1 β (10 ng/mL) (B). SZP secretion was quantified by enzyme-linked immunosorbent assay (ELISA). All individual treatment groups were not significantly different according to a 2-way analysis of variance (ANOVA) with a Tukey-Kramer honestly significant difference *post hoc* test ($P > 0.07$). However, as a factor, both the anabolic cytokine TGF- β 1 ($P < 0.02$), and catabolic cytokine IL-1 β ($P < 0.05$), were significantly different than their respective No Cytokine factors. KGN (10 μ M) as a factor was not significantly different than control (0 μ M KGN) for the TGF- β 1 ($P > 0.82$) or IL-1 β experiments ($P > 0.12$). Values are presented as mean $\pm$ SD ($n = 8$).

accumulation significantly ($P < 0.003$) compared with No Growth Factor groups (0 μ M KGN, 0.04 ± 0.02 ; 10 μ M KGN, 0.03 ± 0.01 μ g/mL) at Day 4 of culture, KGN had no effect ($P > 0.89$) on SZP synthesis from synoviocytes (Fig. 5B). The ELISA results were corroborated by immunoblot analysis (Fig. 5A).

Discussion

The objective of this study was to determine the effects of KGN on SZP secretion in primary, superficial zone articular chondrocytes under different culture methods, and to investigate potential interactions between KGN and regulatory

Figure 5. The effects of kartogenin (KGN), transforming growth factor- β 1 (TGF- β 1) on synoviocytes. Primary, monolayer cultures of bovine, synoviocytes were cultured in serum-free, defined media supplemented with and without KGN (10 μ M), in the presence and absence of TGF- β 1 (3 ng/mL). The media was changed at day 4, and the cultures were terminated at day 7. According to an analysis of the day 4 media via enzyme-linked immunosorbent assay (ELISA) and 1-way analysis of variance (ANOVA) with Tukey-Kramer honestly significant difference *post hoc* test (**B**), TGF- β 1 significantly upregulated ($P < 0.003$) superficial zone protein (SZP) media accumulation. KGN had no apparent effect ($P > 0.89$) on SZP synthesis. Representative SZP immunoblots (**A**) corroborated the ELISA results. Groups not connected by the same letter were determined to be significantly different. Values are presented as mean $\pm$ SD ($n = 6$).

cytokines such as TGF- β 1 and IL-1 β . Using immunoblot and ELISA, KGN was determined to have no significant effect on altering SZP synthesis in superficial zone chondrocytes cultured in monolayer, micromass, and explant cultures. Synoviocytes, the other major contributor of SZP to boundary lubricants in the synovial fluid, were similarly unaffected by KGN. While bioactivity was confirmed through changes in SZP mRNA expression, KGN did not modulate SZP protein synthesis in combination with TGF- β 1 or IL-1 β treatments either. As KGN had no effect on SZP synthesis in primary, superficial zone articular chondrocytes, this evidence suggests that the observed, chondrogenic effects of KGN are dependent on cellular differentiation status.

Kartogenin was identified in 2012 as a small, heterocyclic molecule capable of stimulating chondrogenesis in primary, human MSCs.²¹ Chondrogenic activity was also observed in bovine articular cartilage explants, where KGN was found to reduce GAG release into media.²¹ Most interestingly, Johnson and colleagues²¹ noted that KGN enhanced the gene expression of lubricin/superficial zone protein. These observations motivated this current investigation to examine the response of SZP protein synthesis to KGN

treatment in primary, bovine, superficial zone articular chondrocytes. In combination with TGF- β 1, KGN enhanced gene expression of SZP in monolayer cultures at concentrations of 100 nM and 1 μ M (**Fig. 2B**). However, this increased gene expression did not lead to any changes in protein synthesis, as measured by SZP secretion in monolayer (**Fig. 2A**), micromass (**Fig. 3A**), and cartilage explant (**Fig. 4**) cultures. The corresponding data from these three different *in vitro* culture systems suggest that the culture system, or cell context, is an unlikely contributor to the observed results. Likewise, equivalent results were obtained from both predominant producers of SZP in the synovial joint, superficial zone articular chondrocytes (**Fig. 2A**) and synoviocytes (**Fig. 5**). In light of the relative maturity of the cells examined, the chondrogenic effects of KGN may depend on cell naivety, with cell responsiveness to KGN decreasing with cellular differentiation and maturation. This inference regarding the effectiveness of KGN in relation to the potency and differentiation status of a cell will require additional study.

The dissimilar responses to KGN from progenitor and differentiated cells have been observed elsewhere in the literature. Decker and coworkers³³ observed increased GAG

accumulation in micromass cultures of embryonic, pre-skeletal MSCs in response to KGN. Additionally, KGN augmented GAG synthesis in leporine MSCs and patellar tendon stem/progenitor cells.³⁴ However, Ono *et al.*²² found no changes in GAG from KGN treatment in tissue engineered, neocartilage disks produced from adult, bovine articular chondrocytes. Following this same pattern, KGN upregulated aggrecan, collagen II, and Sox9 expression in leporine MSCs.³⁴ However, in alignment with our results (Fig. 2C-E), in human and bovine articular chondrocytes, KGN-induced changes in the expression of these genes were not observed by Ono *et al.*²² Investigating these cell differentiation state-dependent effects of KGN may yield important insights into KGN's mechanism of action.

SZP/lubricin/PRG4 is gaining prominence as a marker of articular cartilage. In addition to collagen II, aggrecan, and Sox9, investigations in the literature frequently assay SZP gene expression as an indicator of the chondrogenic phenotype. Indeed, SZP is a hallmark of the superficial zone of articular cartilage as it functions as a boundary lubricant, reducing friction and wear at the articular surface.⁶ However, it is important to note that SZP is a marker at the glycoprotein level, and needs to be measured as such. While middle and deep zone articular chondrocytes express the *prg4* gene,^{35,36} these zonal phenotypes do not synthesize and secrete measurable amounts of the glycoprotein.^{7,37} The expression of *prg4* in middle and deep zone articular chondrocytes has another important implication; mRNA expression levels of SZP do not correlate to protein synthesis. While this general concept is well established,³⁸⁻⁴⁰ the results of this investigation (Fig. 2) and previous studies demonstrate that it applies to SZP as well.¹⁴ In summary, meaningful measurements of SZP/lubricin/PRG4 must be performed at the glycoprotein level as its mRNA expression values do not necessarily correspond to protein synthesis.

In conclusion, the story of KGN appears to be incomplete as much remains to be elucidated and described. KGN did not stimulate SZP synthesis in synoviocytes, and superficial zone articular chondrocytes cultured under multiple conditions. Based on this investigation, and others described in the literature, the chondrogenic effects of KGN appear to be attenuated in differentiated cell phenotypes, such as articular chondrocytes. However, these apparent differences between progenitor and differentiated cell types in response to KGN merit additional investigation since important new, mechanistic insights into the effects of KGN may be revealed.

Acknowledgments and Funding

The authors thank Dr. Thomas M. Schmid of Rush University for his gift of the antibody S6.79. This investigation was supported by the Lawrence J. Ellison Chair in Musculoskeletal Molecular Biology at the University of California, Davis, and the National Institute of Arthritis and Musculoskeletal and Skin Diseases at the National Institutes of Health under Award Number AR061496. The content is solely the responsibility of the authors and does not

necessarily represent the official views of the National Institutes of Health.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Ethical approval was not sought for the present study because neither living animals, nor humans, nor human tissues were used in the conduct of this research.

Animal Welfare

Guidelines for humane animal treatment did not apply to the present study because no living animals were involved. All tissues were obtained from an abattoir.

References

- Centers for Disease Control and Prevention. Prevalence and most common causes of disability among adults—United States, 2005. *MMWR Morb Mortal Wkly Rep.* 2009;58:421-6.
- Charnley J. The lubrication of animal joints in relation to surgical reconstruction by arthroplasty. *Ann Rheum Dis.* 1960;19:10-9.
- Mow VC, Ratcliffe A, Poole AR. Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures. *Biomaterials.* 1992;13:67-97.
- Becerra J, Andrades JA, Guerado E, Zamora-Navas P, Lopez-Puertas JM, Reddi AH. Articular cartilage: structure and regeneration. *Tissue Eng Part B Rev.* 2010;16:617-27.
- Jones BA, Pei M. Synovium-derived stem cells: a tissue-specific stem cell for cartilage engineering and regeneration. *Tissue Eng Part B Rev.* 2012;18:301-11.
- Schumacher BL, Block JA, Schmid TM, Aydelotte MB, Kuettner KE. A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch Biochem Biophys.* 1994;311:144-52.
- Niikura T, Reddi AH. Differential regulation of lubricin/superficial zone protein by transforming growth factor β /bone morphogenetic protein superfamily members in articular chondrocytes and synoviocytes. *Arthritis Rheum.* 2007;56:2312-21.
- Swann DA, Hendren RB, Radin EL, Sotman SL, Duda EA. The lubricating activity of synovial fluid glycoproteins. *Arthritis Rheum.* 1981;24:22-30.
- Schmidt TA, Sah RL. Effect of synovial fluid on boundary lubrication of articular cartilage. *Osteoarthritis Cartilage.* 2007;15:35-47.
- Jay GD, Harris DA, Cha CJ. Boundary lubrication by lubricin is mediated by O-linked β (1-3)Gal-GalNAc oligosaccharides. *Glycoconj J.* 2001;18:807-15.
- Marcelino J, Carpten JD, Suwairi WM, Gutierrez OM, Schwartz S, Robbins C, *et al.* CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome. *Nat Genet.* 1999;23:319-22.

12. Rhee DK, Marcelino J, Baker M, Gong Y, Smits P, Lefebvre V, *et al.* The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth. *J Clin Invest.* 2005;115:622-31.
13. Coles JM, Zhang L, Blum JJ, Warman ML, Jay GD, Guilak F, *et al.* Loss of cartilage structure, stiffness, and frictional properties in mice lacking PRG4. *Arthritis Rheum.* 2010;62:1666-74.
14. Lee SY, Niikura T, Reddi AH. Superficial zone protein (lubricin) in the different tissue compartments of the knee joint: modulation by transforming growth factor β 1 and interleukin-1 β . *Tissue Eng Part A.* 2008;14:1799-808.
15. Khalafi A, Schmid TM, Neu C, Reddi AH. Increased accumulation of superficial zone protein (SZP) in articular cartilage in response to bone morphogenetic protein-7 and growth factors. *J Orthop Res.* 2007;25:293-303.
16. Hattori S, Oxford C, Reddi AH. Identification of superficial zone articular chondrocyte stem/progenitor cells. *Biochem Biophys Res Commun.* 2007;358:99-103.
17. DuRaine G, Neu CP, Chan SM, Komvopoulos K, June RK, Reddi AH. Regulation of the friction coefficient of articular cartilage by TGF- β 1 and IL-1 β . *J Orthop Res.* 2009;27:249-56.
18. Chan SM, Neu CP, Duraine G, Komvopoulos K, Reddi AH. Atomic force microscope investigation of the boundary-lubricant layer in articular cartilage. *Osteoarthritis Cartilage.* 2010;18:956-63.
19. Peng G, McNary SM, Athanasiou KA, Reddi AH. The distribution of superficial zone protein (SZP)/lubricin/PRG4 and boundary mode frictional properties of the bovine diarthrodial joint. *J Biomech.* 2015;48:3406-12.
20. Lee SY, Nakagawa T, Reddi AH. Mesenchymal progenitor cells derived from synovium and infrapatellar fat pad as a source for superficial zone cartilage tissue engineering: analysis of superficial zone protein/lubricin expression. *Tissue Eng Part A.* 2010;16:317-25.
21. Johnson K, Zhu S, Tremblay MS, Payette JN, Wang J, Bouchez LC, *et al.* A stem cell-based approach to cartilage repair. *Science.* 2012;336:717-21.
22. Ono Y, Ishizuka S, Knudson CB, Knudson W. Chondroprotective effect of kartogenin on CD44-mediated functions in articular cartilage and chondrocytes. *Cartilage.* 2014;5:172-80.
23. Kang ML, Ko JY, Kim JE, Im GI. Intra-articular delivery of kartogenin-conjugated chitosan nano/microparticles for cartilage regeneration. *Biomaterials.* 2014;35:9984-94.
24. Decker RS, Koyama E, Enomoto-Iwamoto M, Maye P, Rowe D, Zhu S, *et al.* Mouse limb skeletal growth and synovial joint development are coordinately enhanced by kartogenin. *Dev Biol.* 2014;395:255-67.
25. Andrades JA, Motaung SC, Jimenez-Palomo P, Claros S, Lopez-Puerta JM, Becerra J, *et al.* Induction of superficial zone protein (SZP)/lubricin/PRG 4 in muscle-derived mesenchymal stem/progenitor cells by transforming growth factor- β 1 and bone morphogenetic protein-7. *Arthritis Res Ther.* 2012;14:R72.
26. Chen P, Carrington JL, Hammonds RG, Reddi AH. Stimulation of chondrogenesis in limb bud mesoderm cells by recombinant human bone morphogenetic protein 2B (BMP-2B) and modulation by transforming growth factor β 1 and β 2. *Exp Cell Res.* 1991;195:509-15.
27. Nugent-Derfus GE, Takara T, O'Neill J K, Cahill SB, Gortz S, Pong T, *et al.* Continuous passive motion applied to whole joints stimulates chondrocyte biosynthesis of PRG4. *Osteoarthritis Cartilage.* 2007;15:566-74.
28. Su JL, Schumacher BL, Lindley KM, Soloveychik V, Burkhart W, Triantafillou JA, *et al.* Detection of superficial zone protein in human and animal body fluids by cross-species monoclonal antibodies specific to superficial zone protein. *Hybridoma.* 2001;20:149-57.
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods.* 2001;25:402-8.
30. Lee SY, Nakagawa T, Reddi AH. Induction of chondrogenesis and expression of superficial zone protein (SZP)/lubricin by mesenchymal progenitors in the infrapatellar fat pad of the knee joint treated with TGF- β and BMP-7. *Biochem Biophys Res Commun.* 2008;376:148-53.
31. Schmidt TA, Gastelum NS, Han EH, Nugent-Derfus GE, Schumacher BL, Sah RL. Differential regulation of proteoglycan 4 metabolism in cartilage by IL-1 α , IGF-I, and TGF- β 1. *Osteoarthritis Cartilage.* 2008;16:90-7.
32. Jones AR, Flannery CR. Bioregulation of lubricin expression by growth factors and cytokines. *Eur Cell Mater.* 2007;13:40-5.
33. Decker RS, Koyama E, Enomoto-Iwamoto M, Maye P, Rowe D, Zhu S, *et al.* Mouse limb skeletal growth and synovial joint development are greatly enhanced by kartogenin. *Dev Biol.* 2014;395:255-67.
34. Zhang J, Wang JH. Kartogenin induces cartilage-like tissue formation in tendon-bone junction. *Bone Res.* 2014;2:14008.
35. Amanatullah DF, Yamane S, Reddi AH. Distinct patterns of gene expression in the superficial, middle and deep zones of bovine articular cartilage. *J Tissue Eng Regen Med.* 2012;8:505-14.
36. Darling EM, Athanasiou KA. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J Orthop Res.* 2005;23:425-32.
37. Schmidt TA, Schumacher BL, Klein TJ, Voegtline MS, Sah RL. Synthesis of proteoglycan 4 by chondrocyte subpopulations in cartilage explants, monolayer cultures, and resurfaced cartilage cultures. *Arthritis Rheum.* 2004;50:2849-57.
38. Chen G, Gharib TG, Huang CC, Taylor JM, Misek DE, Kardias SL, *et al.* Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics.* 2002;1:304-13.
39. Halbeisen RE, Gerber AP. Stress-dependent coordination of transcriptome and translome in yeast. *PLoS Biol.* 2009;7:e1000105.
40. Uemura N, Kondo T. Current advances in esophageal cancer proteomics. *Biochim Biophys Acta.* 2015;1854:687-95.