Interactive Cytokine Regulation of Synoviocyte Lubricant Secretion

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Cytokine regulation of synovial fluid (SF) lubricants, hyaluronan (HA), and proteoglycan 4 (PRG4) is important in health, injury, and disease of synovial joints, and may also provide powerful regulation of lubricant secretion in bioreactors for articulating tissues. This study assessed lubricant secretion rates by human synoviocytes and the molecular weight (MW) of secreted lubricants in response to interleukin (IL)-1 β , IL-17, IL-32, transforming growth factor-beta 1 (TGF- β 1), and tumor necrosis factor-alpha (TNF- α), applied individually and in all combinations. Lubricant secretion rates were assessed using ELISA and binding assays, and lubricant MW was assessed using gel electrophoresis and Western blotting. HA secretion rates were increased ~40-fold by IL-1 β , and increased synergistically to ~80-fold by the combination of IL-1 β + TGF- β 1 or TNF- α + IL-17. PRG4 secretion rates were increased ~80-fold by TGF- β 1, and this effect was counterbalanced by IL-1 β and TNF- α . HA MW was predominantly <1 MDa for controls and individual cytokine stimulation, but was concentrated at >3 MDa after stimulation by IL-1 β + TGF- β 1 + TNF- α to resemble the distribution in human SF. PRG4 MW was unaffected by cytokines and similar to that in human SF. These results contribute to an understanding of the relationship between SF cytokine and lubricant content in health, injury, and disease, and provide approaches for using cytokines to modulate lubricant secretion rates and MW to help achieve desired lubricant composition of fluid in bioreactors.

Introduction

THE SYNOVIAL JOINT is a low-friction, low-wear load-bearing system that includes articular cartilage, synovial fluid (SF), and synovium components. Articular cartilage is composed of chondrocytes within a dense extracellular matrix, and bears load and slides relative to an apposing surface with low-friction and low-wear properties. The SF acts as a biomechanical lubricant for articular cartilage, as it contains a number of lubricating molecules as well as regulatory cytokines, nutrients, and other components. The SF lubricant molecule hyaluronan (HA) is produced predominantly by fibroblast-like synoviocytes in synovium (abbreviated here as synoviocytes),^{1,2} whereas the lubricating molecule proteoglycan 4 (PRG4) is secreted by synoviocytes as well as chondrocytes in the superficial layer of articular cartilage.^{3–6} The synthesis rate of these lubricating molecules and the molecular weight (MW) of molecules synthesized are important because the balance between the rate of synthesis and the rate of loss of these molecules, by degradation and transport through the semipermeable synovium, dictates their concentration in SF.^{7,8}

In naturally occurring or animal models of osteoarthritis (OA), rheumatoid arthritis (RA), and injury, the concentra-

tions of HA and PRG4 in SF are often decreased, as is the MW of HA.^{9–13} However, the volume of SF is often increased to an even greater extent,^{9,14,15} resulting in an overall increase in lubricant content in SF, and complicating assessments of lubricant production and degradation. These changes in lubricant content and volume in SF are often accompanied by increases in the concentrations of certain cytokines in SF, including interleukin (IL)-1 β , IL-17, IL-32, transforming growth factor-beta 1 (TGF- β 1), and tumor necrosis factor-alpha (TNF- α).^{16–25} The individual effects of some of these cytokines on lubricant secretion by synoviocytes have been studied,^{1,26–28} but interactive effects that likely occur *in vivo* remain to be established. A combinatorial approach to evaluate the interactive effects of cytokines *in vitro* could provide a better understanding of cytokine-regulated lubricant secretion *in vivo*.

To gain further understanding of such *in vivo* regulation, and to generate lubricious fluid using a biomimetic approach, we developed a bioreactor system for evaluating the production and regulation of SF lubricants²⁹ (Fig. 1). Other experimental culture systems have been developed to include certain features of the synovial joint to examine the biology and mechanobiology of synovium and cartilage^{30,31},

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FIG. 1. Synovial joints may be modeled theoretically with a compartmental approach, and experimentally in a bioreactor with key components that include lubricant-secreting cell types, a SF compartment, and a lubricant-retaining semi-permeable membrane. Chemical and mechanical stimuli are key parameters that may regulate lubricant secretion and MW, and affect lubricant composition in native and bioengineered SF. MW, molecular weight; SF, synovial fluid. Color images available online at www.liebertonline.com/ten.

however, such systems have not attempted to generate or study a fluid with lubricant properties similar to that of native SF. In the biomimetic bioreactor system, application of cytokines may provide insight into *in vivo* regulation of SF that occurs in healthy and diseased joints, and may also provide modulation of fluid composition by powerfully regulating lubricant secretion. The ability to generate a lubricating fluid in such a bioreactor system could help to develop arthritis therapies, such as novel viscosupplements.³²

As a step toward developing such a bioreactor to evaluate the production and regulation of lubricants in SF, we evaluated here the regulatory effects of cytokines commonly implicated in arthritis.^{16–25} Specifically, we examined the secretion rates of HA and PRG4 by human synoviocytes, as well as the MW of these secreted molecules, as regulated by the cytokines IL-1 β , IL-17, IL-32, TGF- β 1, and TNF- α applied individually and in combination. Using this information, we can tailor the cytokine milieu in future bioreactor studies to mimic normal synovium and arthritis.

Materials and Methods

Synoviocyte and synovial fluid isolation

Human synovial tissue and SF samples were collected with IRB approval and informed donor consent. All studies were approved by the Human Subjects Research Protection Program of the University of California–San Diego.

Synovial tissue was obtained from patients with OA or RA at the time of joint replacement, and synoviocytes were isolated from the tissue. The diagnosis of RA conformed to the 1987 revised American College of Rheumatology criteria. As described previously,²⁰ synovial tissues from both RA and OA sources were minced and incubated with 1 mg/mL collagenase in serum-free Dulbecco's Modified Eagle Medium (DMEM) with additives (100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL Fungizone, 0.1 mM Modified Eagle Medium (MEM) nonessential amino acids, 10 mM HEPES, 0.4 mM L-proline, and 2 mM L-glutamine) for 2 h at 37°C, filtered through a nylon mesh, extensively washed, and cul-

tured in DMEM supplemented with 10% fetal bovine serum (FBS). As native synovium and synovial tissue digests are generally a heterogeneous mixture of cell types (fibroblast-like synoviocytes, macrophage-like synoviocytes, blood cells, etc.), fibroblast-like lubricant-secreting synoviocytes were enriched by removing nonadherent cells after overnight culture, and by subsequent trypsinization and passaging of cells (split at a 1:3 ratio and cultured in DMEM + 10% FBS). Synoviocytes were used from passages 3 through 9 when they are a homogeneous population of fibroblast-like synoviocytes (<1% CD11b, <1% phagocytic, and <1% Fc γ RII positive).

Normal human SF was obtained from the normal knee (as assessed by X-ray) of subjects with intra-articular fractures in the contralateral knee or ankle, with informed consent. Collected SF was clarified of cells and debris by centrifugation (3000 g, 30 min, 4°C), and resultant samples were stored at –70°C before analysis.

Synoviocyte culture and cytokine treatment

For all experiments, synoviocytes were seeded at 10,000 cells/cm² in standard multiwell tissue culture plates and cultured as two-dimensional monolayers in DMEM + 10% FBS until confluent. Confluent synoviocytes were subsequently incubated under basal conditions of DMEM + 0.5% FBS for 2 days. This period of serum starvation suppresses proliferation and basal mediator production while permitting a robust response to exogenous cytokines. Cells were then cultured for an additional 3 days in DMEM + 0.5% FBS (control) $\pm$ cytokines, applied either individually or in different combinations. All subsequent analyses were performed on either cell layers or conditioned media during the 3 day cytokine treatment. Donor tissue for each experiment consisted of six different donors, with three OA sources (66 $\pm$ 20 years, six donors) and three RA sources (60 $\pm$ 11 years, six donors). As the effects of individual and combinations of cytokines did not statistically differ for OA and RA groups in the analyses, data are presented and discussed collectively.

Treatment with individual cytokines. Individual cytokines were applied over a range of concentrations to assess dose responses: IL-1 β , IL-17, IL-32, TGF- β 1 (0.1, 1, and 10 ng/mL), and TNF- α (1, 10, and 100 ng/mL). Concentrations in these ranges have been previously shown to regulate aspects of synoviocyte biosynthesis, such as production of IL-6, IL-8, and granulocyte macrophage colony stimulating factor (GM-CSF).^{16–21,25} IL-1 β , IL-17 (isoform IL-17A), TGF- β 1, and TNF- α were purchased from Peprotech (Rocky Hill, NJ), and IL-32 (isoform not assessed) was purchased from Abnova (Taipei City, Taiwan). Endotoxin levels for cytokines were <0.1 ng/ μ g (1 EU/ μ g).

Treatment with combinations of cytokines. A fully factorial experimental design of cytokine combinations (32 groups total) was used to elucidate additive and/or synergistic effects of IL-1 β , IL-17, IL-32, TGF- β 1 (10 ng/mL), and TNF- α (100 ng/mL). In preliminary experiments, certain combinations of cytokines at high concentrations (10 ng/mL for IL-1 β , IL-17, IL-32, and TGF- β 1, and 100 ng/mL for TNF- α), but not low concentrations (0.1 ng/mL for IL-1 β , IL-17, IL-32, and TGF- β 1, and 1 ng/mL for TNF- α), resulted in marked synergistic regulation of lubricant secretion; thus, all experiments in this study were carried out using the high cytokine concentrations.

Additionally, as different donor tissue was utilized for the different experiments (i.e., individual cytokine dose response and combinations of cytokines), individual cytokine conditions at the high cytokine concentrations were included in both experiments.

DNA content

Net cell proliferation was assessed as the ratio of DNA on day 3 of cytokine treatment to DNA on day 0 of cytokine treatment. Cell layers were solubilized on days 0 and 3 with 0.5 mg/mL proteinase K (Roche, Indianapolis, IN), harvested, and analyzed for DNA using PicoGreen® (Molecular Probes, Eugene, OR).³³

Lubricant secretion rates

Secretion of HA and PRG4 was assessed after the 3 day cytokine treatment by analyzing conditioned medium for PRG4 by ELISA using mAb GW4.23 (gift from Dr. Klaus Kuettner),³⁴ and for HA by an enzyme-linked binding assay using HA binding protein.³⁵ Secretion rates (r_{HA} and r_{PRG4}) were determined by normalizing total mass of secreted lubricant to cell number on day 3 as estimated from DNA, and to culture duration.

Molecular weight distribution of secreted HA

The MW distribution of HA secreted by synoviocytes was performed using an agarose gel electrophoresis and Stainsall detection technique,³⁶ with several modifications. Conditioned medium samples (pooled from six donors for each treatment condition, and subsequently from individual donors for conditions that exhibited a marked regulatory effect to allow statistical analysis), normal human SF, and HA standards in the MW range 0.16–4.0 MDa (Associates of Cape Cod, East Falmouth, MA) were all treated with or without *Streptomyces* hyaluronidase (10 U/mL) (Seikagaku, Tokyo, Japan) overnight at 37°C, and then subsequently with proteinase K (0.5 mg/mL) overnight at 37°C to eliminate interference of protein in the assay. Samples with HA mass of 200–500 ng were applied to 1% agarose gels (Lonza, Rockland, ME), separated by horizontal electrophoresis at 100 V for 110 min in TAE buffer (0.4 M Tris-acetate and 0.01 M ethylenediaminetetraacetic acid, pH 8.3), and observed after incubation with 0.1% Stainsall reagent (Sigma, St. Louis, MO). Gel images were digitized with a D80 digital camera (Nikon, Melville, NY) and also processed to determine HA distribution by subtracting the intensities of hyaluronidase-treated samples from that of nonhyaluronidase-treated samples, and then displaying on a relative scale (0 to 1) to show relative HA content between 0.1 and 7.0 MDa. Also, the distribution of HA within the MW ranges of 0.1–1, 1–3, and >3 MDa was calculated.

Molecular weight of PRG4

The MW of PRG4 secreted by synoviocytes was determined for samples showing upregulation by Western blot utilizing mAb GW4.23 to PRG4³⁴ or nonspecific mouse IgG as a control. Conditioned medium was concentrated with a 30 kDa MWCO centrifugal filtration device (Millipore, Billerica, MA), and then these samples (0.15 µg PRG4 equivalent) and also normal human SF (0.5 µL) were treated with 10 U/mL *Streptomyces* hyaluronidase (Seikagaku) overnight

at 37°C. Samples were then applied to precast 3–8% acrylamide Tris-acetate gels, and separated by electrophoresis at 150 V for 1 h in Tris-acetate sodium dodecyl sulfate running buffer (50 mM Tricine, 50 mM Tris base, and 0.1% sodium dodecyl sulfate, pH 8.24), blotted onto polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ) at 30 V for 1 h in transfer buffer (25 mM Bicine, 25 mM Bis-Tris, and 1 mM ethylenediaminetetraacetic acid, pH 7.20). The membranes were then blocked for 1 h in 5% normal goat serum in phosphate-buffered saline + 0.1% Tween 20 (pH 7.4), reacted with mAb GW4.23 to PRG4 (or nonspecific mouse IgG as a control [Pierce, Rockford, IL]) at 0.5 µg/mL in 1% bovine serum albumin in phosphate-buffered saline + 0.1% Tween 20 for 1 h, and then a goat anti-mouse secondary antibody conjugated to horseradish peroxidase. Immunoreactivity was detected by ECL-Plus chemiluminescence (Amersham Biosciences), which was recorded with a Storm Imager (GMI, Ramsey, MN).

Statistical analysis

Data are expressed as mean ± standard error of the mean. Data were log transformed to improve normality. For individual cytokines, effects on DNA and secretion rates were analyzed by one-way analysis of variance (ANOVA) and Dunnett's *post hoc* test to assess differences from controls. For combinations of cytokines, a five-way ANOVA (factors: IL-1β, IL-17, IL-32, TGF-β1, and TNF-α) was used to assess interactive (nonadditive) effects of cytokines on DNA and secretion rates. Tukey *post hoc* tests assessing the differences between each cytokine combination were also performed

FIG. 2. Regulatory effects of the individual cytokines IL-1β, IL-17, IL-32, TGF-β1, and TNF-α at a range of concentrations on (A) proliferation, (B) HA secretion, and (C) PRG4 secretion by human synoviocytes. Mean ± SEM, $n = 5-6$; $*p < 0.05$. HA, hyaluronan; PRG4, proteoglycan 4; IL, interleukin; TGF-β1, transforming growth factor-beta 1; TNF-α, tumor necrosis factor-alpha; SEM, standard error of the mean.

(see Supplemental Materials, available online at www.liebertonline.com/ten). For analysis of HA MW, effects of cytokines were analyzed by one-way ANOVA, and Tukey *post hoc* test to assess differences among samples in the percentage of total HA present in MW ranges of <1, 1–3, and >3MDa.

Results

DNA analysis

Overall synoviocyte proliferation was low during the 3 day cytokine treatment. The minimal relative proliferation was consistent with the high cell density of monolayers on the initial day of cytokine treatment and the short culture duration. There was a slight stimulatory effect of TGF- β 1, IL-1 β , and TNF- α ($p < 0.05$) (Figs. 2A and 3A).

Lubricant secretion rates

r_{HA} and r_{PRG4} were differentially regulated by individually applied cytokines in a dose-dependent manner (Fig. 2B, C). The r_{HA} of control cultures was increased ~22-fold by IL-1 β ($p < 0.05$). Less stimulatory to r_{HA} were TNF- α and TGF- β 1, causing increases to ~4.2- and ~2.8-fold, respectively (each, $p < 0.05$) (Fig. 2B). In contrast, r_{PRG4} was affected only by TGF- β 1, being increased to ~78-fold over the control rate ($p < 0.05$) (Fig. 2C). r_{HA} and r_{PRG4} were not significantly affected by the addition of IL-17 or IL-32 individually.

Synergistic interactions of certain combinations of cytokines increased r_{HA} markedly over the additive effects of individual cytokines (Fig. 3B). TNF- α acted synergistically with several other cytokines, including IL-1 β , IL-17, and TGF- β 1, increasing r_{HA} to ~1.3-, ~6.3-, and ~2.3-fold over the predicted additive effects of these cytokines, respectively (each, $p < 0.05$). IL-1 β also acted synergistically with IL-17 and with TGF- β 1, increasing r_{HA} ~1.2- and ~2.1-fold over the predicted additive effects (each, $p < 0.05$). r_{HA} was not significantly affected by addition of IL-32 to any other individual cytokine or combination of cytokines.

PRG4 secretion was modulated by cytokines, with counterbalancing effects of certain combinations (Fig. 3C). The stimulatory effect of individually applied TGF- β 1 was counterbalanced by addition of either IL-1 β or TNF- α , decreasing r_{PRG4} significantly ($p < 0.05$). When applied together with TGF- β 1, IL-1 β and TNF- α further decreased r_{PRG4} . r_{PRG4} was unchanged by addition of IL-17 or IL-32 to any condition.

Effect of cytokines on lubricant molecular weight

The MW distribution of secreted HA was markedly regulated by individual and combinations of cytokines, and resembled that of normal human SF in some cases. For all samples, hyaluronidase treatment eliminated detection of signal in agarose gels, indicating that staining of the protease-digested samples was specific for HA (Fig. 4A). The percentage of total HA present in the MW ranges of >3, 1–3, and <1MDa for all cytokine conditions using samples pooled

FIG. 3. Regulatory effects of different combinations of the cytokines IL-1 β , IL-17, IL-32, TGF- β 1, and TNF- α (applied at maximum individual stimulation doses) on (A) proliferation, (B) HA secretion, and (C) PRG4 secretion by human synoviocytes. Mean $\pm$ SEM, $n = 6$; * $p < 0.05$ for individual effect; ** $p < 0.05$ for interactive effect. (+) signs indicate cytokines present in the culture media.

FIG. 4. (A) Observation of HA in conditioned medium samples from selected regulatory cytokine treatments in the MW range of 0.1–7.0 MDa, using agarose gel electrophoresis technique. Plus (+) signs indicate the negative control samples that were subjected to HA digestion with *Streptomyces* hyaluronidase. (B) Percentage of total HA in the MW ranges of >3, 1–3, and <1 MDa, averaged from relative intensities of samples in (A), mean $\pm$ SEM, $n = 2-4$; * $p < 0.05$ versus both control and TGF- $\beta 1$; # $p < 0.05$ versus IL-1 β .

from multiple donors revealed an overall regulation by the cytokines IL-1 β , TGF- $\beta 1$, and TNF- α , applied individually and in combination (Fig. 5). Selected regulatory conditions that were further analyzed from individual donors (Fig. 4B) showed that for control and TGF- $\beta 1$ conditions, >90% of HA had a MW below 1 MDa. It should be noted that as IL-17 and IL-32 appeared to induce a similar low-MW profile for HA, these conditions were not statistically analyzed. For individually applied IL-1 β , the MW profile of HA was more diffuse, with a larger percentage of HA in the 1–3 MDa range (~30% for IL-1 β vs. ~5% for control and ~3% for TGF- $\beta 1$, $p < 0.05$) and also in the >3 MDa range (~11% for IL-1 β vs. ~2% for control and ~1% for TGF- $\beta 1$, $p < 0.05$), although the majority of HA was still present at <1 MDa. The effect of TGF- $\beta 1$ in combination with IL-1 β shifted the MW distribution of HA to a higher percentage in the >3 MDa range (~34% vs. ~11% for IL-1 β , $p < 0.05$). The effect of TNF- α in combination with TGF- $\beta 1$ and IL-1 β did not significantly alter the MW distribution from that of TGF- $\beta 1$ + IL-1 β , but showed a trend of increasing percentage of HA in the >3 MDa range. This trio combination of cytokines generated a MW profile that most closely resembled that of normal human SF (~70% in the >3 MDa range).

The secreted PRG4 had a high MW that did not appear to be differentially regulated by any combination of cytokines analyzed, and also appeared similar to that present in normal human SF (Fig. 6).

Discussion

This study examined the regulatory effects of selected cytokines, individually and in combination, on synovioocyte HA and PRG4 secretion. HA secretion rates were increased ~40-fold by IL-1 β and to a lesser extent by TNF- α , and increased synergistically to ~80-fold by the combination of IL-1 β + TGF- $\beta 1$ or TNF- α + IL-17. PRG4 secretion rates were increased ~80-fold by TGF- $\beta 1$, with counterbalancing effects by IL-1 β and TNF- α . The MW of secreted HA was distributed primarily in the <1 MDa range for controls and after stimulation by individual cytokines, but was distributed in the >1 MDa range and concentrated at >3 MDa after stimulation by IL-1 β + TGF- $\beta 1$ + TNF- α and resembled the distribution in normal human SF. The MW of secreted PRG4 was unaffected by cytokines and similar to that present in normal human SF. The results of this study contribute to an understanding of the relationship between SF cytokine and lubricant content in health, injury, and disease, and provide approaches for using cytokines to modulate lubricant secretion rates and MW to achieve desired lubricant composition of fluid generated in joint bioreactors.

The interpretation of the data from the present study may apply to synovioocytes isolated from a normal joint, although the synovioocytes utilized in this study were isolated from synovium of patients undergoing joint replacement due to either OA or RA. In using these cells, we note that most functions of late passage synovioocytes isolated from normal, OA, and RA synovium have similar responses to cytokines, including proliferation, metalloproteinase production, adhesion molecule expression, and activation of intracellular signaling pathways.³⁷ Although some modest differences have been observed in the invasion into cartilage *in vivo*, these properties were not examined in the present studies. These considerations, along with difficulty obtaining well-characterized fibroblast-like synovioocytes from normal individuals, led us to focus on cells derived from total joint replacement samples.

The effects of individually applied cytokines extend previous studies on regulation of synovioocyte HA and PRG4 secretion and provide key information for development of an *ex vivo* bioreactor to study SF biology. The observed stimulatory effects on HA secretion of individually applied IL-1 β , TGF- $\beta 1$, and TNF- α , and the synergistic effects of IL-1 β and TGF- $\beta 1$ have been previously demonstrated.^{1,26,27} Similarly, the observed stimulatory effects of TGF- $\beta 1$ on PRG4 secretion, and the counterbalancing effects of IL-1 β and TNF- α are consistent with the reported individual effects of these cytokines.^{4,38,39} The demonstrated effect of IL-1 β to induce secreted HA to be distributed at higher MWs over basal controls also confirms previous reports,^{1,28} as does the observed high MW of PRG4 secreted with TGF- $\beta 1$ stimulation.⁴

The current study provides new information on the synergistic effects of TNF- α and multiple cytokines on HA secretion. Although synovioocyte synthesis of certain molecules, including IL-6, IL-8, and GM-CSF, is synergistically stimulated by interaction of TNF- α with other cytokines,^{16,19,21} this study is the first report of such interactive regulation of lubricant secretion. The synergistic effects of cytokines on HA secretion would likely lead to an increase in total HA content in native SF, with effects on concentration that are dependent on fluid volume. The volume of SF in normal

FIG. 5. (A) Relative HA intensity profiles in the MW range of 0.1–7.0 MDa for all conditioned medium samples (pooled from $n = 6$) and normal human SF. (B) Percentage of total HA in the MW ranges of >3, 1–3, and <1 MDa, calculated from relative intensities of samples in (A). (+) signs indicate cytokines present in the culture media. Color images available online at www.liebertonline.com/ten.

knee joints is ~ 1 –2 mL, but increased ~ 10 –25-fold in inflamed or diseased joints^{9,14,15}; in the latter case, HA concentration is decreased, but only to 30–50% of normal levels.^{9,11,13} Thus, the total mass of HA in SF (volume multiplied by concentration) is typically substantially increased with joint inflammation or disease, and could be a result of upregulation of synovioocyte secretion, mediated by certain cytokines. The synergistic effect of IL-17 with TNF- α suggests that T cells secreting IL-17 could play a role in the regulation of SF lubricant composition, whereas the interactive effects of TNF- α with not only IL-17, but also IL-1 β and TGF- β 1 suggest that therapies targeting TNF- α ^{40,41} might be more potent than expected based on the individual effects of TNF- α .

FIG. 6. Observation of PRG4 from conditioned medium with selected individual and combination cytokine treatments and also human SF, using techniques of gel electrophoresis, Western blotting, and chemiluminescent detection. (+) signs indicate cytokines present in the culture media.

There are a number of mechanisms by which cytokines could have regulated HA and PRG4 secretion as observed in this study. Marked increases in mRNA expression of a specific HA synthase (HAS) enzyme, HAS1 (rather than HAS2 or HAS3), occurs in response to IL-1 β , TGF- β 1, or TNF- α , applied individually,⁴² and certain intracellular signaling molecules, including MAP kinases, have been identified in the regulation of such expression.^{42,43} The HAS enzymes might also underlie the regulation of HA size, as HAS3-transfected fibroblasts synthesized HA of lower MW than HAS2- and HAS1-transfected cells.⁴⁴ Other potential mechanisms of cytokine regulation of HA include catabolism mediated by HA-degrading hyaluronidases,⁴⁵ cellular trafficking of HAS proteins, activation of sugar nucleotide HA building blocks, interactions between HAS enzymes and the nascent growing HA chains as they are extruded from the cell,^{46,47} and interactions between HA and binding proteins such as TSG-6¹⁸ that stabilize HA and prevent its depolymerization in SF.⁴⁸ Signaling mechanisms behind PRG4 regulation have been studied less extensively than that of HA, but have suggested the role of Smad2/3 in mediating TGF- β 1 signaling.⁴⁹ Although these regulatory mechanisms of HA and PRG4 secretion by individual cytokines have been identified, it is unknown whether regulation by cytokines in combination occurs through signaling pathways that are distinct or involve crosstalk. Further analyses of such signaling pathways may reveal whether certain combinations of cytokines have counterbalancing effects.

The size distribution of HA in SF is dependent not only upon synthesis and degradation processes, but also upon HA retention. As transport of HA out of SF occurs through the voids of the synovium extracellular matrix,^{8,50} alterations in the tissue structure or in the MW of secreted HA might affect the rate that HA permeates the tissue and is lost from SF. The effect of cytokine-regulated alterations in the size distribution of HA may be magnified by size-dependent retention properties of the synovium, with low MW forms of

HA being lost quickly from SF and high MW forms being selectively retained.

The ability of cytokines acting individually or synergistically to regulate synovioocyte HA and PRG4 secretion likely affects the health of articulating joint surfaces *in vivo*, and will be particularly useful in *in vitro* synovial joint bioreactors to modulate the lubricity of the fluid component.³⁰ For example, a ~100-fold increase in HA concentration with certain combinations of cytokines would likely result in increased fluid viscosity, as well as increased binding of lubricants to tissue surfaces, thus enhancing the friction-reducing ability of the fluid. Our subsequent studies have incorporated the information of the current study into experiments to evaluate the proper membranes for synovioocyte adherence and lubricant flux, and to generate a lubricating fluid in a biomimetic bioreactor.²⁹ Although the effects of cytokine combinations on chondrocyte PRG4 secretion and matrix metabolism were not assessed in this study, the reported negative effects of cytokines such as IL-1 β and TNF- α on these processes may be balanced by the positive effects of TGF- β 1. Cytokine combinations that result in imbalances and lubricant deficiencies in fluid generated in synovial joint bioreactors may be useful in mimicking aspects of injury and disease and gaining insight into *in vivo* regulation of SF that may occur in pathological conditions.

The results of this study provide information on cytokine regulation of HA and PRG4 secretion rates and MW, and contribute to an understanding of the relationship between alterations in SF cytokine and lubricant content that occur in injury and disease. The results also provide approaches for using cytokines in bioreactors to modulate lubricant secretion rates and MW over a range of magnitudes. Application of this knowledge may allow achievement of desired lubricant composition of fluid generated in bioreactors with articulating tissues. The ability to generate a fluid with modulated lubricant composition could also facilitate development of arthritis therapies, such as viscosupplements or molecules that regulate lubricant secretion.

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Disclosure Statement

The authors declare that no competing financial interests exist.

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