Normal and Osteoarthritic Human Synovial Fluid Regulation of Articular Cartilage Proteoglycan-4 Secretion: Role of O_2 and TGF-β

1. BACKGROUND

Osteoarthritis (OA) is a degenerative disease that results in destructive changes to joint structures including cartilage, synovium, and bone (1). It is the most common joint disorder in the world, being one of the most frequent causes of pain in Western populations. A majority of people 65 years of age have radiographic evidence of OA, while this increases to 80% in individuals over 75 years of age (2). The prevalence of OA in aging individuals is so high that it is often regarded as an inevitable consequence of aging. As the population of the United States ages, it is becoming increasingly important that this disease be better understood.

Synovial fluid in non-diseased states consists of a variety of macromolecules, including plasma proteins, lubricant molecules (including PRG4), growth factors (including TGF-β1), and cytokines (1). PRG4 is one of the two primary lubricating macromolecules in synovial fluid. Depending on the detection method, PRG4 levels in normal human synovial fluid (hSF) have been reported to have a broad range from a concentration of 35 μg/ml to 250 μg/mL (3, 4). TGF-β1 generally upregulates PRG4 gene expression and protein secretion (5). With the addition of 10 ng/mL to the culture medium, TGF-β1 stimulates the secretion of PRG4 by 2-10–fold (6). The concentration of lubricant in SF reflects a balance between secretion and loss. Thus, one way in which PRG4 and TGF-β1 concentrations in synovial fluid may be related is through variable TGF-β1 regulation of PRG4 secretion by chondrocytes.

The reported concentrations of expression of PRG4 and TGF-β1 in synovial fluid have varied levels and may depend on injury and disease states of the joint. In OA hSF, PRG4 levels have been found to be decreased to 147±28 μg/ml, compared with a normal PRG4 concentration of 287±32 μg/ml (3). In addition, the concentrations of PRG4 and TGF-β1, along with other macromolecules in synovial fluid, appear to be highly variable in knee injury (1).

Some chondrocyte functions depend on oxygen tension. Normal articular cartilage is in a hypoxic environment. Normal SF, which supplies the oxygen that diffuses into articular cartilage, has a low oxygen tension of 50–70 mmHg (7). At the articular surface, oxygen tension is about 10% (7). As chondrocytes in the more superficial layers consume oxygen, the oxygen tension in the deepest layers of cartilage are <1% (7).

Chondrocytes are typically cultured at 20% oxygen. However, this does not mimic the environment *in vivo*. Several studies have examined the effect of oxygen tension on chondrocyte viability using different measures, including trypan blue exclusion, extracellular matrix production (7), GAG production (8), and mRNA levels (7). Although chondrocytes were able to survive in all oxygen tensions, anoxic conditions resulted in less metabolically active chondrocytes, resulting in decreased matrix production, rRNA, and mRNA (7). Ysart *et al*., 1994, found that 21% oxygen resulted in the highest amount of GAG production (8), using bovine explants from 1-2 year old steers.

2. RATIONALE

2.A. CULTURE PARAMETERS - %O_2, OA SF viscosity, %OA SF

Some chondrocyte functions are dependent on oxygen tension. While chondrocytes are typically cultured at 20% oxygen, this is not the most physiologically relevant environment. A pilot study was conducted to determine several parameters for optimal detection of changes in PRG4 secretion as well as the sample size required for a subsequent full-scale study. In this pilot study, effects of oxygen tension, OA SF viscosity type, and percent OA SF were examined.

As OA is a spectrum disease, there are many different ways in which SF can be altered in OA, resulting in variations in the color, viscosity, and amount of SF present. Chondrocytes were cultured in both high and low viscosity to assure there was not an effect of viscosity. Based on the CTE SF collection, there were 118 samples of high viscosity OA SF and 77 samples of low viscosity OA SF.

A dose response of SF was conducted in order to ensure minimal amount of SF was used while still yielding a sufficient response in PRG4 secretion to detect any changes based on parameters laid out in the experimental groups (**Table 3**).

2.B. EFFECTS OF SF (NL & OA), TGF-β1, LY2157299

SF contains biochemical factors that can regulate cartilage in health or disease. SF composition, which includes cytokines and growth factors, can vary with different joint states. SFs from different joint states may differentially regulate cartilage, mediated by different concentrations and activities of biochemical factors.

Cartilage PRG4 secretion can be regulated by a number of biochemical factors. Two of the most powerful are TGF-β1, which stimulates PRG4 secretion, and IL-1α/IL-1β, which inhibit PRG4 secretion. The effects of these cytokines and growth factors that are endogenous to human synovial fluid on cartilage PRG4 secretion, however, is unknown. The purpose of the full experiment was to determine the effects of TGF-β1 on PRG4 secretion in acute injury and osteoarthritis synovial fluid and attempt to block these effects with a TGF-β1 inhibitor LY2157299 using an optimal concentration of the inhibitor as determined by the dose response study.

3. HYPOTHESIS

3.A. CULTURE PARAMETERS - %O_2, OA SF viscosity, %OA SF

(1) Higher oxygen tension will result in higher PRG4 secretion by chondrocytes. (2) Higher synovial fluid concentration will result in higher PRG4 secretion, as synovial fluid has been found to have a stimulatory effect on PRG4 secretion.

3.B. EFFECTS OF SF (NL & OA), TGF-β1, LY2157299

(1) Chondrocytes cultures in OA SF will have higher PRG4 secretion than basal and NL SF. (2) The addition of TGF-β1 inhibitor LY2157299 to culture containing OA SF have PRG4 secretion levels similar to levels from culture in NL SF.

4. OBJECTIVES

4.A. CULTURE PARAMETERS - %O_2, OA SF viscosity, %OA SF

To determine the effects of culture conditions (%O_2) and OA SF sample type and dose (low or high viscosity OA SF, at 2.5%, 5%, 10%, or 20% SF) on PRG4 secretion.

4.B. EFFECTS OF SF (NL & OA), TGF-β1, LY2157299

To determine the effects of NL and OA hSF with and without TGF-β1 and TGF-β1 inhibitor on PRG4 secretion.

5. STUDY DESIGN & APPROACH

5.A. CULTURE PARAMETERS - %O_2, OA SF viscosity, %OA SF

Bovine explants from the lateral femoral condyle (LFC) and medial femoral condyle (MFC) were examined under various conditions. There were 31 experimental groups studied (**Table 2**). Cartilage was examined in various conditions: 1) fresh or 2) incubated in medium 2.A) alone, with 2.B) TGF-β1 or 2.C) OA SF, of 2.C.1) high or 2.C.2) low viscosities, at different oxygenation levels. The effects of % OA SF (2.5, 5, 10, 20%), OA SF viscosity (high or low), and oxygenation level (5, 10, 20%) were determined and compared to fresh cartilage, and cartilage incubated in basal medium (negative control) or in medium with TGF-β1 (positive control). Cartilage was incubated in medium, 0.160 ml / disk, collected and replaced with fresh medium every 2 days. Collected conditioned medium was stored at -80 °C. Portions of which were be analyzed for PRG4 (d4 – 6 conditioned medium).

5.B. EFFECTS OF SF (NL, OA), TGF-β1, LY2157299

Bovine explants from the LFC and MFC were examined under various conditions: 1) fresh or 2) incubated in medium 2.A) alone, or 2.B) 5% SF from either 2.B.1) NL donors, or 2.B.2) OA donors with and without 2.C) TGF-β1 and with and without 2.D) TGF-β1 inhibitor LY2157299 (1 μM) (**Table 3**). The effect of 5% NL and OA SF, with and without 10 ng/ml TGF-β1, and with and without LY2157299 on PRG4 secretion will be determined, and cartilage incubated in basal medium (negative control) or in medium with TGF-β1 (positive control). Cartilage will be incubated in medium, 0.160 ml / disk, collected and replaced with fresh medium every 2 days. Collected conditioned medium will be stored at -80 °C. Four calf knees were used, each of which received a different NL and OA SF donor (**Table 1**).

5.C. hSF SAMPLES

The following hSF samples were selected. Half were male and half were female from each group, except the injury group (Table 1). The average age of OA SF donors was 62.5±2.1, and the average age of the NL SF donors was 71.5±11.2. Age and sex matching was attempted, but due to the low availability of NL samples with sufficient volume, this was not possible. For OA samples, SF that fit the criteria of straw color, clear clarity, and low viscosity were selected. Low viscosity SF was used as it has been found to be more typical of OA SF (9). The rationale for this was that OA SF characteristically has low concentrations of hyaluronic acid compared to normal SF, and hyaluronic acid is primary determinant of SF viscosity (10). SF from donors with plateau fractures were used. Half the samples were red, indicating blood is present. These samples were chosen as they were representative of acute injury. Half the samples were straw colored, to attempt to negate the effects blood may have on PRG4 secretion. NL SF samples were selected to be colorless, clear, and high viscosity, as this is most typical of normal SF, based on the synovial fluids present in the Cartilage Tissue Engineering Synovial Fluid database. Samples were selected which had no history or evidence of joint disease.

DonorID	Group (OA, NL)	Age	Sex	Side	Color	Clarity	Viscosity	Vol Remain [ml]
	CCB2013-01							
WDB307	OA	66	F		Straw	Clear	Low	12.0
	CCB2013-03							
09-087	NL	55	F		Colorless	Clear	High	0.6
WDB375	OA	61	M		Straw	Clear	Low	8.2
	CCB2013-06							
10-009	NL	75	F		Colorless	Clear	High	0.9
WDB330	OA	64	F	L	Straw	Clear	Low	9.2
	CCB2013-08							
10-039	NL	76	M		Colorless	Clear	High	0.5
WDB327	OA	60	M		Straw	Clear	Low	1.5
	CCB2013-09							
10-041	NL	80	M		Colorless	Clear	High	0.5
WDB385	OA	65	F		Straw	Clear	Low	1.2

Table 1: SF samples used for experiments.

5.C. EXPERIMENTAL GROUPS

5.C.1. CULTURE PARAMETERS - $\%O_2$, OA SF viscosity, %OA SF

#	rhTGF-β1 [ng/ml]	%SF	SF Viscosity	$\%O_2$	# disks
1	-	-	-	-	7
2	0	0	-	5	3
3	10	0	-	5	3
4	0	2.5	Low	5	2
5	0	5	Low	5	2
6	0	10	Low	5	2
7	0	20	Low	5	3
8	0	2.5	High	5	2
9	0	5	High	5	2
10	0	10	High	5	2
11	0	20	High	5	3
12	0	0	-	10	3
13	10	0	-	10	3
14	0	2.5	Low	10	2
15	0	5	Low	10	2
16	0	10	Low	10	2
17	0	20	Low	10	3
18	0	2.5	High	10	2
19	0	5	High	10	2
20	0	10	High	10	2
21	0	20	High	10	3
22	0	0	-	20	3
23	10	0	-	20	3
24	0	2.5	Low	20	2
25	0	5	Low	20	2
26	0	10	Low	20	2
27	0	20	Low	20	3
28	0	2.5	High	20	2
29	0	5	High	20	2
30	0	10	High	20	2
31	0	20	High	20	3

Table 2. Experimental Groups for culture parameter study

5.C.2. Effects of SF (NL & OA), TGF-β1, LY2157299

#	Group	TGF-β1 [ng/mL]	TGF-β1 inhibitor [μM]	Synovial Fluid	# disks / knee
1	Basal	0	0	None	6
2	TGFβ1	10	0	None	6
3	Inh	0	1	None	6
4	TGFβ1+Inh	10	1	None	6
5	NL SF	0	0	5% NL	2
6	OA SF	0	0	5% OA	2
7	NL SF+Inh	0	1	5% NL	2
8	OA SF+Inh	0	1	5% OA	2
9	NL SF+TGFβ1	10	0	5% NL	2
10	NL SF+TGFβ1+Inh	10	1	5% NL	2
11	OA SF+TGFβ1	10	0	5% OA	2
12	OA SF+TGFβ1+Inh	10	1	5% OA	2
				Total:	42

Table 3: Experimental groups for the study of the effects of SF types, TGF-β1, and LY2157299 on PRG4 secretion by calf explants. Controls (Groups 1-4), SF ± inhibitor on PRG4 secretion (Groups 5-16). Groups 1-4 had three explants from the LFC (Groups#A, B, C), and three from the MFC (Group#D, E, F). Groups 5-16 had one explant from the LFC (labeled Group#A) and one explant from the MFC (labeled Group#B). Four experiments (CCB2013-03, CCB2013-06, CCB2013-08, CCB2013-09) were completed with four calf knees and SF from different donors (**Table 1**).

6. METHODS

6.A. OA & NL SF Preparation

Synovial fluid was obtained from NL & OA knees of adult humans as previously described (11, 12). For NL knees, cadaveric knee blocks were obtained from tissue banks and tapped within 48 h of death to obtain undiluted fluid. Following IRB-approved human subjects protocols, undiluted synovial fluid was obtained from subjects who gave consent and were undergoing either total knee arthroplasty for OA (OA-hSF) or undergoing surgery after sustaining a closed tibial plateau fracture. All fluids were centrifuged to separate out cells and debris, and the supernatants aliquotted and frozen at -80 °C. Samples have been selected for use in the proposed studies, matching the age and sex of NL and OA specimens.

Neat OA and NL SF was diluted 1:20 with basal medium, resulting in 5% OA SF by volume. 5% SF was then filtered with a 0.45 μm syringe filter. A portion of 5% SF, not incubated with calf explants, was saved for analysis by PRG4 ELISA.

6.B. Cartilage Explant Harvest

2 mm diameter disks were isolated from lateral femoral condyles (LFC) or medial femoral condyles (MFC) of immature (1–3 week old) bovine stifle joints, by scoring articular cartilage with a 2 mm diameter dermal punch, then undercutting with a #10 scalpel blade to release cartilage explants with ~2 mm thickness. 2 mm diameter disks were then shaved to 0.5 mm thickness, containing the superficial zone, using slicing jigs. For each experimental group, half of the explants used were from the LFC, and half from MFC (for specific information on which disk was harvested from which location, see description above **Tables 3**).

6.C. Culture Conditions

To assess regulation of chondrocyte secretion of PRG4 secretion, explant cultures of cartilage were incubated in medium under various culture conditions (**Table 3**) and assessed for levels of newly-synthesized PRG4. Cartilage explants were cultured individually in 96 well plates. Freshly harvested cartilage explants were first pre-cultured overnight (16 hours) in basal medium (0.01% BSA, DMEM, 0.1 mM non-essential amino acids solution, 10 mM HEPES, 0.4 mM L-proline, 2 mM L-glutamine, 25 ug/ml ascorbate, Antibiotic-antimycotic 100 units/ml penicillin G, streptomycin sulfate, and amphotericin B) at 10% O_2. Disks were then incubated for 6 days in medium, with or without 10 ng/ml TGF-β1 (Peprotech: #100-21C), in NL or OA SF, and with or without TGF-β1 inhibitor (LY2157299). Medium was changed every two days.

6.D. Inhibitor Preparation

LY2157299 was weighed out to 1 mg and diluted in DMSO to yield a 10 mM stock solution. The stock solution was filtered using a 0.2 micro filter, diluted and added directly to the medium, with a maximum concentration of DMSO of 0.1%. Additional DMSO was added to yield a final concentration of 0.1% in all conditions.

6.E. Analyses

6.E.1. Live/dead

Cartilage disks of fresh explants immediately after harvesting and one disk from each condition in **Table 3** were analyzed for cell viability in the first main experiment. Group 1-4 in **Table 3** were subsequently analyzed for viability for the additional main experiments.

6.E.2. Bovine PRG4 ELISA

Bovine PRG4 standards were from conditioned medium from bovine explants incubated in DMEM, 0.01% BSA, and 10 ng/ml TGF-β1. PRG4 secretion by calf bovine articular cartilage explants incubated in basal medium was 1.3 μg / [cm^2 • day], and with TGF-β1 stimulation, 65 μg / [cm^2 • day]. Conditioned medium was analyzed by PRG4 ELISA at 2x, 10x, and 50x dilutions. For most experimental groups, samples at 10x dilution were sufficiently dilute such that SF will not be a major interferent, yet not too dilute such that PRG4 in medium will be above the limits of sensitivity.

6.F. Statistics

PRG4 secretion data was log-transformed to improve homoscedasticity. Data are expressed as mean±SEM, n=# of cartilage disks, and m=# of donors per condition. PRG-4 secretion data were log-transformed prior to statistical analysis. The effects of various stimuli on PRG-4 secretion was assessed by ANOVA with human SF donor as a random factor and stimulus as a repeated factor, with comparisons to basal conditions and/or with/without inhibitor by t-test with Bonferroni correction as appropriate.

7. RESULTS

O_2 and OA-hSF regulate cartilage PRG-4 secretion. Cartilage PRG-4 secretion was modulated by O_2 ($p < 0.01$) and OA-hSF ($p < 0.01$) (**Fig. 1**). At 5% O_2, PRG-4 secretion was different compared to at 10% O_2 ($p < 0.05$) and 20% O_2 ($p < 0.01$), but secretion at 10% O_2 and 20% O_2 was similar ($p = 0.96$). At 10% O_2, explants secreted higher PRG-4 ($p < 0.001$) with 2.5% OA-hSF compared to without (6.6 ± 3.9 vs. 0 μg/[cm^2·day]), but was similar ($p = 0.63–0.82$) to 5–20% (6.7 ± 2.3, 6.8 ± 3.7, 5.4 ± 2.6 μg/[cm^2·day] at 5%, 10%, 20%, respectively). Thus, 10% O_2 and hSF up to 5% were used in subsequent experiments.

OA-hSF, but not HS, stimulates cartilage PRG-4 secretion. Cartilage PRG-4 secretion was stimulated by OA-hSF ($p < 0.001$), with a trend for NL-hSF ($p = 0.14$) and no effect of HS ($p = 0.49$) (**Fig. 2**). Explants incubated with 5% NL-hSF, OA-hSF, or HS secreted PRG-4 at levels intermediate that of explants incubated in medium alone (2.3 ± 0.6 μg/[cm^2·day]) and those in medium with 10 ng/ml TGF-β1 (21.5 ± 3.1 μg/[cm^2·day]). Explants in 5% OA-hSF secreted substantially higher ($p < 0.01$) PRG-4 compared to basal, while explants incubated in 5% NL-hSF ($p = 0.26$) and 5% HS ($p = 0.99$) did not.

LY2157299 inhibition of TGF-β1-stimulated cartilage PRG-4 secretion is dose-dependent. The TGF-β1-stimulated secretion of PRG-4 was inhibited in a dose-dependent manner by LY2157299 (**Fig. 3**). LY2157299 at both 1 and 10 μM lowered PRG-4 secretion from stimulated levels (each, $p < 0.05$) to basal levels ($p=0.35–0.61$). Thus, LY2157299 at 1 μM was used in subsequent studies.

LY2157299 inhibits TGF-β1-stimulated cartilage PRG4-secretion in the presence of hSF. Cartilage PRG4 secretion was modulated by hSF ($p < 0.05$), TGF-β1 ($p < 0.01$), and LY2157299 ($p < 0.01$) (**Fig. 4**). TGF-β1-stimulated PRG4 secretion by explants was lowered by 1 μM LY2157299, whether in medium alone ($p < 0.001$), NL-hSF ($p < 0.01$), or OA-hSF ($p < 0.001$), back to non-stimulated levels ($p = 0.21–0.75$).

8. DISCUSSION

In order to determine the optimal culture parameters, a pilot study was conducted in which bovine chondrocyte explants were cultured in various oxygenation levels, various synovial fluid concentrations, and in both high and low viscosity OA SF. Chondrocytes are typically cultured at atmospheric oxygen levels, or 20% oxygen. However, this is not similar to the environment of chondrocytes *in vivo*, as cartilage is a hypoxic tissue (7). Bovine calf explants cultured at 10% and 20% oxygen secreted higher amounts of PRG4 into medium during days 4-6 of culture than those cultured at 5% oxygen (6.4 and 6.3 versus 2.5 μg / [cm^2 * day], respectively) (**Fig 1**). 10% oxygen was chosen for culture conditions, as it was most physiologically relevant, yet resulted in a similar level of PRG4 secretion as 20% oxygenation (**Fig. 1**).

Bovine calf explants were also cultured in 2.5, 5, 10, and 20% synovial fluid to assess the ability of synovial fluid to stimulate PRG4 secretion. Although increasing hSF concentration did increase PRG-4 secretion in 5% O_2, it did not at higher concentrations of O_2 (**Fig. 1**). Thus, 5% SF was chosen in order to maximize the amount of synovial fluid from each donor available while still using an adequate amount to stimulate PRG4 secretion.

Once the culture parameters were selected, four full experiments were conducted using the experimental groups in **Table 3** to assess the role of TGF-β1 in the increased PRG4 secretion seen in OA SF. TGF-β1 is well understood to increase the synthesis and secretion of PRG4 by chondrocyte explants (5, 6). This was validated in the current experiment, with the addition of 10 ng/ml TGF-β1 resulting in significantly higher PRG4 secretion than chondrocytes cultures without TGF-β1. Furthermore, there was a trend in the stimulatory effect of 10 ng/ml TGF-β1 depending on the SF present in medium. The level of PRG4 secretion was higher in chondrocytes stimulated with 10 ng/ml TGF-β1 cultured in 5% OA SF (45.19±12.59 μg / [cm^2 * day]) than for chondrocytes stimulated with 10 ng/ml TGF-β1 cultured in 5% NL SF (31.91±3.32 μg / [cm^2 * day]), as shown in **Figure 4**.

Synovial fluid was also found to have a stimulatory effect on PRG4 secretion into medium by cartilage. Chondrocyte explants incubated in 5% SF were also found to have higher levels of PRG4 secretion compared to those cultured in basal medium (**Fig. 2**). Although 5% NL SF resulted in higher PRG4 secretion than basal medium, the difference between groups was not significant, $p=0.14$. 5% OA SF resulted in significantly higher PRG4 secretion into medium on days 4-6 of culture than basal medium (**Fig. 4**), $p<0.001$.

The ability of the TGF-β1 inhibitor LY2157299 to block the higher PRG4 secretion stimulated chondrocytes cultured in OA SF suggests that TGF-β1 is involved in the higher levels of PRG4 secretion seen in osteoarthritis (**Fig 3**). OA SF has been found to have higher PRG4 levels than NL SF (1). Thus, we concluded that the higher PRG4 secretion by chondrocytes incubated in OA SF was due to stimulation by TGF-β1 in the OA SF.

9. FIGURES

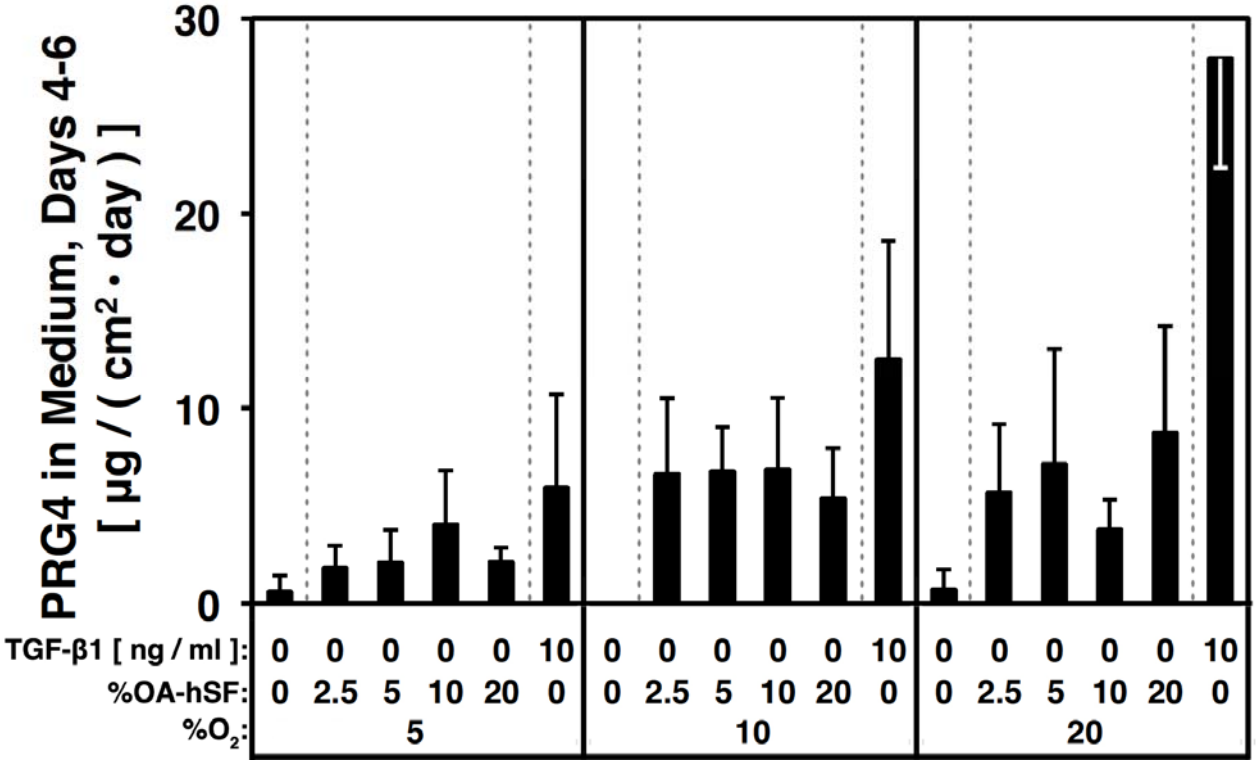

Figure 1: Effects of TGF-β1, OA-hSF, and O_2 on PRG-4 secretion. PRG-4 in conditioned medium from days 4 – 6 of culture of calf explants (n=2 disks per condition) incubated in medium alone, with 10 ng/ml rhTGF-β1, or with varying concentrations (2.5%, 5%, 10%, 20%) of OA-hSF from m=2 patients with osteoarthritis, at either 5%, 10%, or 20% O_2. Mean±SD.

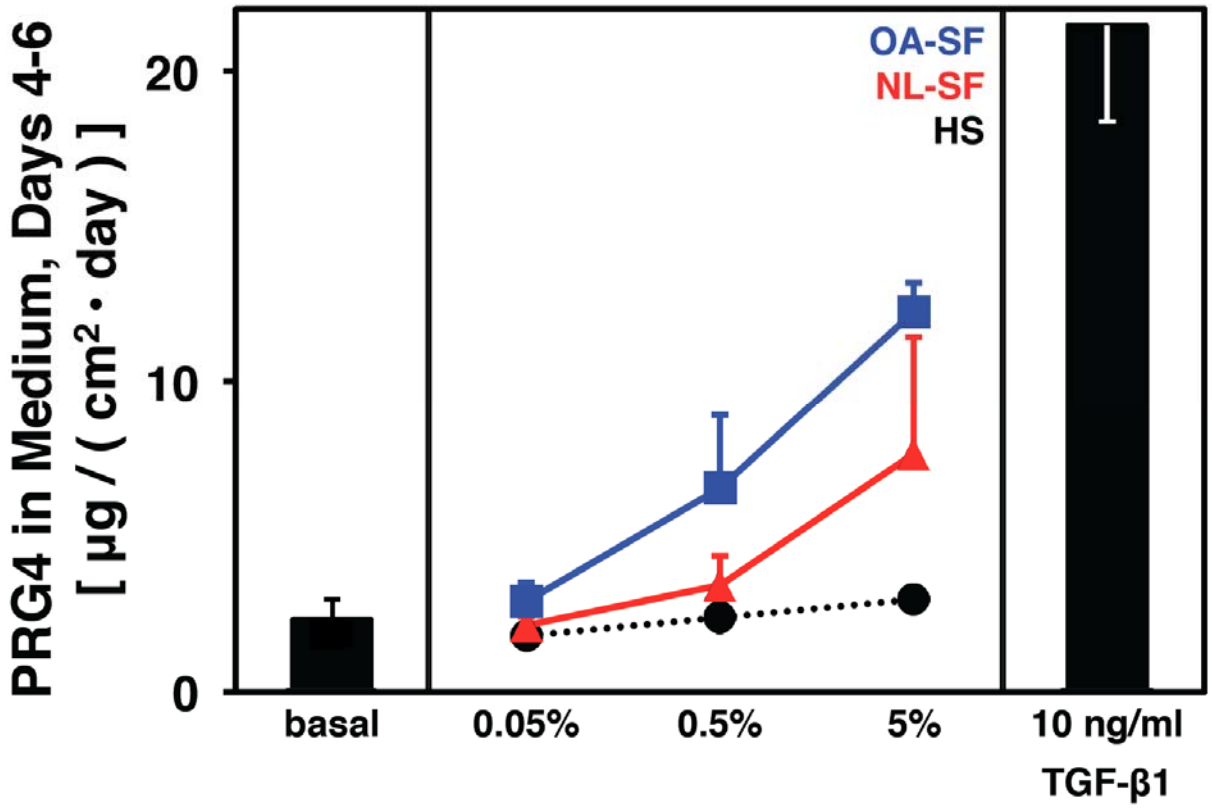

Figure 2: Dose-response of normal and osteoarthritis synovial fluids, and human serum, on PRG-4 secretion. PRG-4 in conditioned medium from days 4 – 6 of culture of calf explants (n=4–6 disks per condition) incubated in medium alone, with 10 ng/ml TGF- β1, or with increasing concentrations (0.05%, 0.5%, 5%) of hSF, either from m=4 normal (NL-hSF, in red) or m=4 osteoarthritic (OA-hSF, in blue) donors, or normal human serum. Pooled human serum was tested on disks from 3 calf knees. Mean±SEM. **p < 0.01 compared to basal condition.

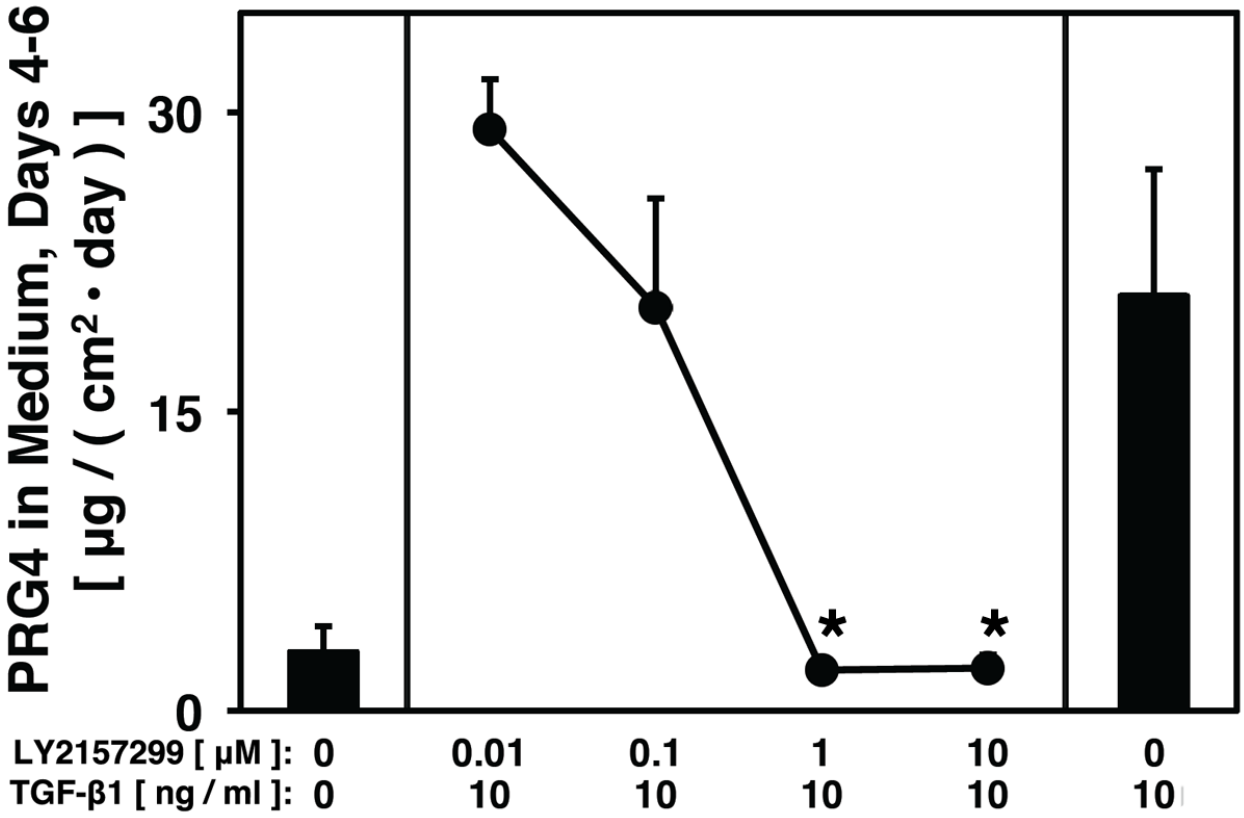

Figure 3: Dose-response of TGFβR1 inhibitor LY2157299 on TGF-β1-stimulated PRG-4 secretion. PRG-4 in conditioned medium from days 4 – 6 of culture of calf explants (n=2–6 disks per condition per knee from 3 calf knees) incubated in medium alone, or with 10 ng/ml TGF- β1, and increasing concentrations (0, 0.01, 0.1, 1, 10 μM) of LY2157299. Mean±SEM. *p < 0.05 compared to TGF- β1 stimulated group without inhibitor.

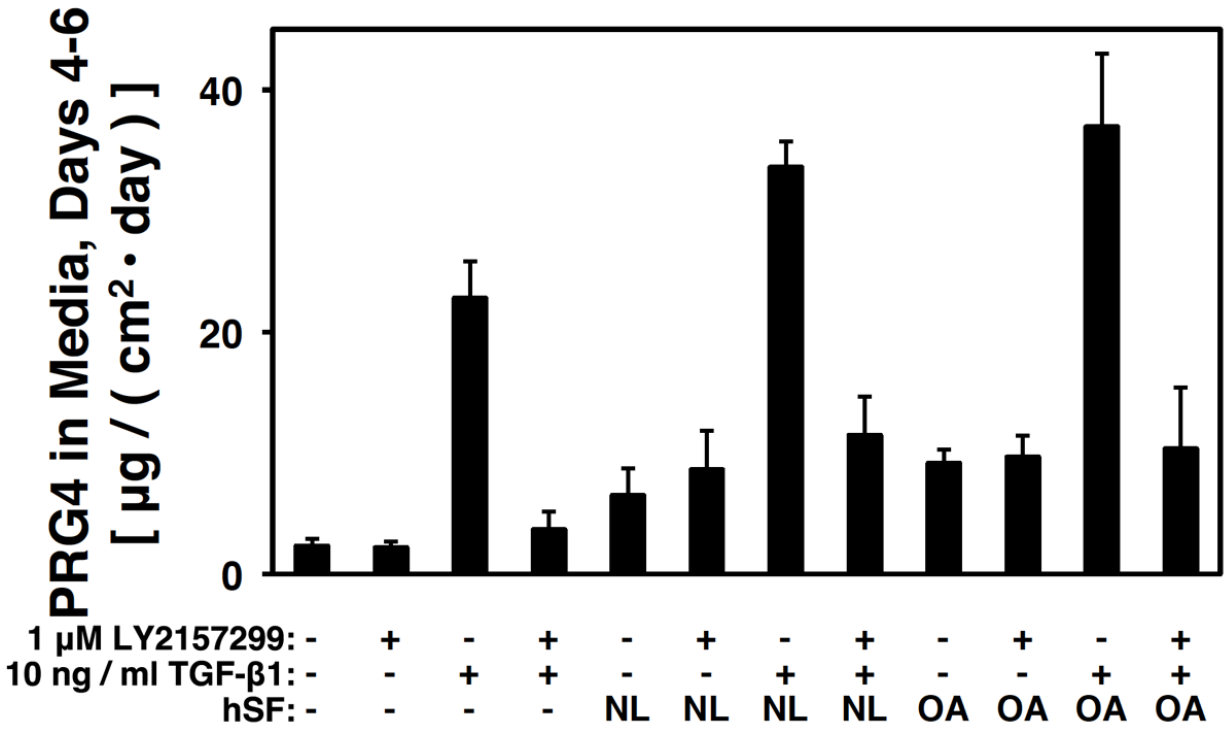

Figure 4: Effects of TGF-β1, LY2157299, and hSF on cartilage PRG4 secretion. PRG-4 in conditioned medium from days 4 – 6 of culture of calf explants (n=2–6 disks per condition per knee from 7 calf knees) incubated in medium ± 10 ng/ml TGF- β1 ± 1 μM LY2157299 ± 5% NL-hSF (m=7 donors or OA-hSF (m=7 donors). Mean±SEM.

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