CMBES/SCGB

2016 CMBEC39 Conference
Calgary AB
May 24–27, 2016

EFFECT OF HYDRODYNAMIC SHEAR ON PROTEOGLYCAN 4 SECRETION BY BOVINE CARTILAGE EXPLANTS

Alyssa Morin[1], Suresh Regmi[2], Tannin Schmidt[1,2,3]
[1]Biomedical Engineering Graduate Program, [2]Faculty of Kinesiology,
[3]Schulich School of Engineering, University of Calgary, AB

INTRODUCTION

Proteoglycan 4 (PRG4), also known as lubricin[1] and superficial zone protein[2], is a mucin-like glycoprotein present in synovial fluid (SF) and at the surface of articular cartilage. PRG4 exists in SF as monomeric and disulfide-bonded multimeric forms, which are secreted by chondrocytes in bovine cartilage explants in vitro[3] and have been shown to be functionally determinant in cartilage boundary lubricating function[4]. Dynamic shear stimulation of cartilage explant cultures has been shown to increase the quantity of high molecular weight (MW) PRG4 species and up-regulate overall PRG4 secretion by 3-4 times that of unloaded controls[5]. Dynamic compression via oscillation of a ceramic ball over a chondrocyte-seeded scaffold has also been shown to increase PRG4 mRNA expression levels[6]. Collectively, these studies demonstrate that mechanical stimuli regulate PRG4 expression. However, the effect of hydrodynamic shear on PRG4 secretion by chondrocytes in vitro remains to be determined. Therefore, the objective of this study was to compare the quantity and immunoreactive size distribution of PRG4 products secreted into medium by chondrocytes in bovine cartilage explants from stirred bioreactor versus T-flask cultures.

METHODS

Cartilage Explant Culture

PRG4 was prepared from a total of 600 cartilage discs (6mm diameter, ~0.3mm thick) harvested from the patellofemoral groove and femoral condyles of 4 skeletally mature bovine stifle joints. 300 cartilage discs were cultured as explants in either a stirred bioreactor (125mL, 90rpm; Shear +) or T-flask with gentle nutation (Shear -) containing Dulbecco's Modified Eagle's Medium (DMEM). Media was supplemented with 0.01% bovine serum albumin, 25ug/mL ascorbic acid and 10ng/mL TGF-β at ~1mL/10^6 cells/day at 37°C in an atmosphere of 5% CO_2. Shear + and Shear - media was collected and replaced every three days over the 27-day culture duration (n=4 independent cultures).

PRG4 Quantity

Shear + and Shear – media aliquots were quantitatively analyzed in duplicate using indirect enzyme-linked immunosorbent assay[7]. Samples were 2x serially diluted, adsorbed overnight, and then reacted with mAb 4D6 (putative N terminal epitope)[8], HRP-conjugated secondary antibody, and TMB substrate with 3 washes PBS-0.1% Tween between each step. PRG4 levels were calculated using a standard of purified full-length recombinant human PRG4 (rhPRG4)[9].

PRG4 Size Distribution

PRG4 species in Shear + and Shear – media, pooled over the 27-day culture duration, were separated using HPLC (Agilent 1260 Infinity Bio-Inert Quaternary LC) with a size exclusion column (Sepax SRT SEC-500, 7.8x300mm) equilibrated in 1X PBS with in-line UV detection. For each run, 50ul of pooled Shear + and Shear – media was injected (n=4) and eluted with a flow rate of 0.5mL/min. UV absorbance at 214nm was monitored and analyzed using OpenLAB CDS ChemStation Edition C.01.07. Peak area ratios for pooled Shear + and Shear – media were calculated by dividing the area of peak 1 by the area of peak 2 (mAu*s), where peak 1 represents disulfide-bonded PRG4 dimers or multimers and

peak 2 represents PRG4 monomers[3]. Peak identities were confirmed using a standard of purified full-length rhPRG4 *(data not shown).*

Pooled Shear + and Shear – media was incubated with (R) or without (NR) NuPAGE sample reducing agent (10x) and heated at 70°C for 10min. Samples were loaded on a Novex 3-8% Tris-Acetate gel with 10ul of HiMark® Pre-Stained High MW Protein Standard. Electrophoresis was conducted in NuPAGE Tris-Acetate SDS running buffer at 4°C for 70min at 150V in a vertical gel apparatus. Gels were subsequently electroblotted to 0.2um nitrocellulose membranes at 200mA for 2hr in NuPAGE Transfer Buffer (0% methanol). Membranes were blocked with 5% non-fat dry milk in TBS-0.1% Tween (TBST) for 60min at room temperature and then incubated overnight at 4°C with rocking with anti-PRG4 mAb 4D6 (0.5ug/mL) (A) or 9G3 (0.02ug/mL; mucin domain epitope)[10] (B) primary antibody in 3% milk/TBST. Membranes were washed in TBST (3x10min), incubated in 0.5ug/mL goat anti-mouse IgG Cy3 (A) or 12.6ng/mL goat anti-mouse IgG HRP (B) secondary antibody in 3% milk/TBST with rocking for 1hr at room temperature. Membranes were washed again in TBST (3x10min) and immunoreactive bands were detected using the GE Typhoon FLA 9500 (A) or SuperSignal West Femto Maximum Sensitivity Substrate and Chemi Genius 2 Bio Imaging System (B).

Statistical Analysis

A Mann-Whitney U-test was used to assess the effect of culture condition on PRG4 accumulation (ug/cm^2/day) in Shear + and Shear - media for each individual collection point (3-day average) and over the 27-day culture duration, with Bonferroni correction. Statistical analysis was implemented using SPSS Statistics 22.0

RESULTS

PRG4 Quantity

The amount of PRG4 accumulated in media (ug/cm^2/day) was consistently higher in stirred bioreactor versus T-flask culture, except for days 0-3, with 3-day average values tending to decrease over the 27-day culture duration (Fig.1). When considering individual collection points, PRG4 accumulation in Shear + media was significantly greater than Shear – media on days 16-18, 19-21, 22-24 and 25-27 (all $p<0.05$). The amount of PRG4 accumulated in Shear + media (11.2±1.7 ug/cm^2/day) was significantly greater than Shear – media (8.88±1.7 ug/cm^2/day) over the 27-day culture duration ($p<0.001$).

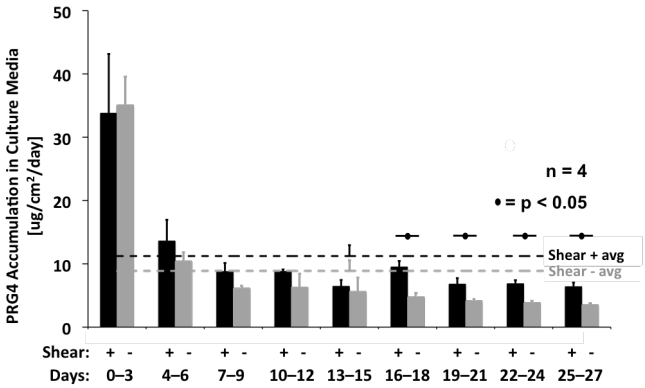

Figure 1: PRG4 accumulation (ug/cm^2/day) in Shear + and Shear – media over the 27-day culture duration. Vales are mean ± SEM.

PRG4 Size Distribution

Two major peaks were observed in the UV trace for pooled Shear + and Shear – media (Fig. 2). Peak 1 represents disulfide-bonded PRG4 dimers and peak 2 represents PRG4 monomers[3]. Calculated peak area ratios, indicative of PRG4 dimer/monomer are shown (Table 1). The ratio of peak 1 to peak 2 is consistently greater for pooled Shear + media compared to pooled Shear – media with mean values of 0.318 and 0.226 respectively.

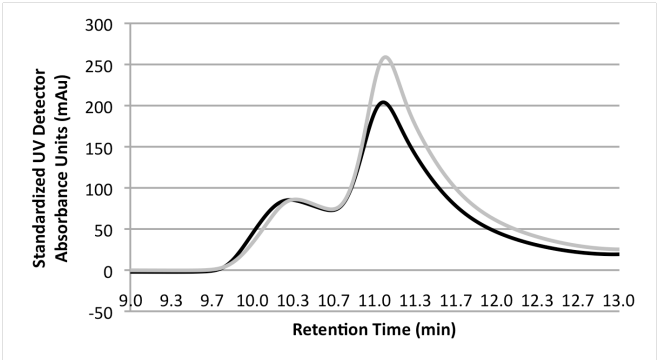

Figure 2: HPCL-SEC chromatogram of pooled Shear + and Shear – media (n=4) showing two peaks of interest.

Table 1: Calculated peak area ratios (dimer/monomer) of pooled Shear + and Shear – media (n=4; A-D).

Culture	Culture Condition	Peak #	Retention Time (min)	Area (mAU*s)	Peak Area Ratio (dimer/ monomer)	Mean Peak Area Ratio (dimer/ monomer)
A	Shear +	1 (dimer)	10.3	3453.3	0.264	0.318 (Shear +)
		2 (monomer)	11.1	13083.9		
	Shear -	1 (dimer)	10.3	3322.1	0.226	
		2 (monomer)	11.1	14676.0		
B	Shear +	1 (dimer)	10.4	2063.7	0.228	
		2 (monomer)	11.1	9067.5		
	Shear -	1 (dimer)	10.4	1975.8	0.164	
		2 (monomer)	11.1	12048.3		
C	Shear +	1 (dimer)	10.3	2109.7	0.323	0.226 (Shear -)
		2 (monomer)	11.1	6537.8		
	Shear -	1 (dimer)	10.3	3244.1	0.277	
		2 (monomer)	11.1	11699.0		
D	Shear +	1 (dimer)	10.3	5995.8	0.423	
		2 (monomer)	11.1	14160.7		
	Shear -	1 (dimer)	10.4	3489.8	0.235	
		2 (monomer)	11.1	14856.8		

Pooled Shear + and Shear – media contained a broad high MW mAb 4D6 immunoreactive species in NR samples, and a single ~460kDa monomeric[3] band (*) in R samples when assessed by SDS-PAGE western blot (Fig.3A). Immunoreactivity to mAb 9G3 resulted in a broad high MW species in NR samples, and a less diffuse band with greater electrophoretic migration at ~460kDa (*) in R samples (Fig.3B).

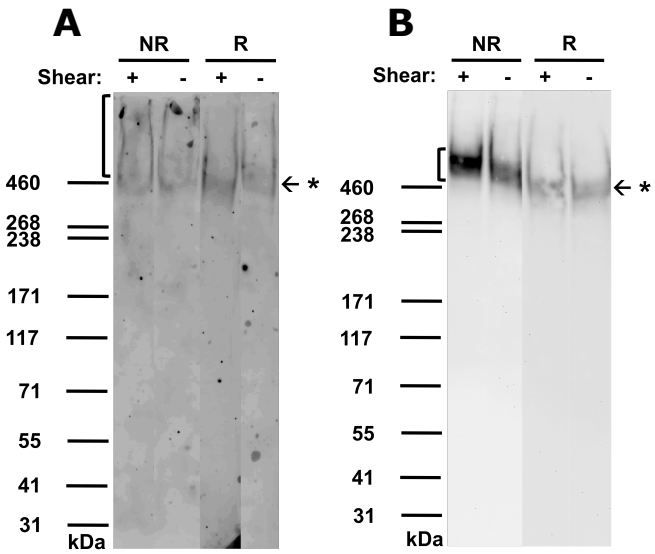

Figure 3: SDS-PAGE western blot of pooled Shear + and Shear – media, NR and R, with anti-PRG4 mAb 4D6 (A) and 9G3 (B).

DISCUSSION

Collectively, these results demonstrate that PRG4 secretion by chondrocytes in vitro is regulated by hydrodynamic shear. Hydrodynamic shear through stirred bioreactor culture, up-regulates PRG4 secretion and the relative ratio of PRG4 dimer/monomer as assessed by HPLC-SEC. Although no detectable effect on immunoreactive size distribution was observed, SDS-PAGE western blotting is a qualitative technique. These results do not contradict the quantitative HPLC-SEC findings, but instead, demonstrate that immunoreactivity to functionally indicative mAb 4D6 and 9G3 is maintained[11,12]. Additional study is required to fully characterize the effect of chemo-mechanical regulation on PRG4 expression in relation to PRG4's disulfide-bonded multimeric form, mucin domain glycosylations and resulting cartilage boundary lubricating function. In conclusion, these results demonstrate that secreted PRG4 quality and quantity is subject to chemo-mechanical regulation, and suggest that PRG4 secretion in vivo could be mechanically regulated by hydrodynamic shear imparted by SF through joint movements.

REFERENCES

[1] D. Swann, H. Slayter, F. Silver, "The molecular structure of lubricating glycoprotein-I, the boundary lubricant for articular cartilage," *The Journal of Biological Chemistry*, vol. 256, pp. 5921-5925, 1981.

[2] B. Schumacher, J. Block, T. Schmid, M. Aydelotte, K. Kuettner, "A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage," *Archives of Biochemistry and Biophysics*, vol. 311, pp. 144-152, 1994.

[3] B. Steele, M. Alvarez-Veronesi, T. Schmidt, "Molecular weight characterization of PRG4 proteins using multi-angle laser light scattering (MALLS)," *Osteoarthritis and Cartilage,* vol. 21, pp. 298-504, 2013.

[4] S. Abubacker, D. Ponjevic, H. Ham, P. Messersmith, J. Matyas, T. Schmidt, "Effect of disulfide bonding and multimerisation on proteoglycan 4's cartilage boundary lubricating ability and adsorption," *accepted to Connective Tissue Research*, 2015.

[5] G. Nugent, N. Aneloski, T. Schmidt, B. Schumacher, M. Boegtline, R. Sah, "Dynamic shear stimulation of bovine cartilage biosynthesis of proteoglycan 4," *Arthritis and Rheumatism,* vol. 54, pp. 1888-1896, 2006.

[6] S. Grad, S. Gogolewski, M. Alini, M. Wimmer, "Effects of simple and complex motion patterns on gene expression of chondrocytes seeded in 3D

scaffolds," *Tissue Engineering,* vol. 12, pp. 3171-3179, 2006.

[7] T. Schmidt, B. Schumacher, T. Klein, M. Voegtline, R. Sah, "Synthesis of proteoglycan 4 by chondrocyte subpopulations in cartilage explants, monolayer cultures and resurfaced cartilage cultures," *Arthritis and Rheumatism,* vol. 50, pp. 2849-2857, 2004.

[8] K. Chawla, H. Ham, T. Nguyen, P. Messersmith, "Molecular resurfacing of cartilage with proteoglycan 4," *Acta Biomaterialia*, vol. 6, pp. 3388-3394, 2010.

[9] S. Abubacker, S. Dorosz, D. Ponjevic, G. Jay, J. Matyas, T. Schmidt, "Full-length recombinant human proteoglycan 4 interacts with hyaluronan to provide cartilage boundary lubrication," *accepted to Annals of Biomedical Engineering,* 2015.

[10] G. Jay, K. Elsaid, K. Kelly, S. Anderson, L. Zhang, E. Teeple, K. Waller, B. Fleming, "Prevention of cartilage degeneration and gait asymmetry by lubricin tribosupplementation in the rat following anterior cruciate ligament transection," *Arthritis and Rheumatism,* vol. 64, pp. 1162-1171, 2012.

[11] T. Schmidt, A. Plass, J. Sandy, "Disulfide-bonded multimers of proteoglycan (PRG4) are present in normal synovial fluids," *Biochimica et Biophysica Acta,* vol. 1790, pp. 375-384, 2009.

[12] G. Jay, K. Waller, "The biology of lubricin: near frictionless joint motion," *Matrix Biology,* vol. 39, pp. 17-24, 2014.