UNIVERSITY OF CALIFORNIA, SAN DIEGO

PROTEOGLYCAN 4 METABOLISM AND BOUNDARY LUBRICATION OF ARTICULAR CARTILAGE

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Bioengineering

by

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The dissertation of Tannin A. Schmidt is approved, and it is acceptable in quality and form for publication on microfilm:

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ABSTRACT OF THE DISSERTATION

PROTEOGLYCAN 4 METABOLISM
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Articular cartilage is the lubricious, load bearing connective tissue at the end of long bones in synovial joints that normally facilitates low-friction and low-wear articulation via a number of lubrication mechanisms. Boundary lubrication is particularly important for the protection and maintenance of the articular surface, which unfortunately often becomes roughened and eroded in aging and arthritis, with the eventual development of pain and dysfunction. Synovial fluid (SF) contains molecules, such as proteoglycan 4 (PRG4) and hyaluronan (HA), that interact with and adsorb to the articular surface, and are therefore ideally positioned to contribute to boundary lubrication. This dissertation aims to contribute to the understanding of

PRG4 metabolism by chondrocytes of the superficial zone of cartilage, and the mechanics of, and contributors to, boundary lubrication of articular cartilage.

PRG4 metabolism by chondrocyte subpopulations in various culture systems was regulated differentially by various biochemical stimuli. The phenotype of PRG4 secretion by chondrocytes was generally maintained in that PRG4 was expressed to a much greater degree by chondrocytes from the superficial zone than by those from the middle and deep zones. PRG4 expression and secretion by chondrocytes near the articular surface was highly inhibited by IL-1α and stimulated by TGF-β1, in a dose-dependent manner, in explant cultures of cartilage.

An *in vitro* articular cartilage-on-cartilage boundary lubrication test was implemented and extended to establish conditions where a boundary mode of lubrication was dominant. SF functioned as an effective friction-lowering boundary lubricant, even with a 3-fold decrease in constituent concentration. SF constituents, PRG4 and HA, contributed individually and in combination, both at physiological and pathophysiological concentrations, to the boundary lubrication of apposing articular cartilage surfaces.

Collectively these results provide insight into the nature of PRG4 metabolism, the biomechanics of boundary lubrication of articular cartilage, and the role of PRG4, alone and in combination with other putative lubricants. The ability to modulate the dynamic regulation of PRG4, whether in homeostasis or degeneration, may ultimately be useful in prolonging the maintenance or slowing the deterioration of articular cartilage's critical mechanical functions at the end of long bones.

CHAPTER 1

INTRODUCTION

1.1 General Introduction to the Dissertation

Articular cartilage is one of several tissues in the body that bears load and slides relative with an apposing tissue surface in a fluid filled cavity. Due to a number of lubrication mechanisms, articular cartilage normally serves over a lifetime as a low-friction, wear-resistant surface in diarthrodial joints. Unfortunately, in aging and arthritis, the articular surface often becomes roughened and eroded, with the eventual development of pain and dysfunction. Such deterioration may be due to repeated excessive loading and/or the failure of lubrication mechanism(s), such as boundary lubrication, which is important for the protection and maintenance of the articular surface. Several molecules synthesized and secreted by cells lining the synovial cavity that are present in synovial fluid (SF), and at the surface of articular cartilage as well, have been implicated as boundary lubricants. These include proteoglycan 4 (PRG4), hyaluronan (HA), and surface active phospholipids (SAPL). *The overall motivation of this dissertation work was to contribute to the understanding of PRG4 metabolism by chondrocytes of the superficial zone of cartilage, and the mechanics of, and contributors to, boundary lubrication of articular cartilage, by examining in vitro*

chondrocyte expression of PRG4 in various culture systems and conditions, and assessing the role of PRG4, HA, and SAPL in the normal boundary lubrication of articular cartilage using an in vitro test.

Chapter 1 begins with a description of the function, structure, and composition of articular cartilage. The posited mechanisms of articular cartilage lubrication are then described, followed by a detailed description of the potential molecular contributors to the boundary lubrication of articular cartilage. Finally, SF composition in health and disease is described.

Chapter 2, which has been published in *Arthritis and Rheumatism* [88], examines the expression of proteoglycan 4 (PRG4) by chondrocyte subpopulations from the superficial, middle, and deep layers in various culture systems. Explant, monolayer, and transplant culture systems were investigated.

Chapter 3, which has been published as a chapter in *Physical Regulation of Skeletal Repair* [87], builds on the results of Chapter 2, and investigates the dose-dependent effects of IL-1α and TGF-β1 on PRG4 metabolism in explant cultures of cartilage in terms of synthesis and secretion by chondrocytes in their native superficial and middle zones, and localization at the articular cartilage surface. Explants were cultured with or without serum, and with various levels of IL-1α or TGF-β1 alone or in sequence.

Chapter 4 extends on the results of Chapter 3 and investigates the effects of IL-1α, IGF-I, and TGF-β1 on PRG4 metabolism in cartilage from the superficial zone, in terms of chondrocyte expression and PRG4 bound at the articular surface. These metabolic indicators are then correlated with PRG4 secretion.

Chapter 5, which has been accepted for publication in *Osteoarthritis and Cartilage*, implements and extend an *in vitro* articular cartilage-on-cartilage lubrication test to elucidate the dependence of the friction properties on sliding velocity, axial load, and time, and establish conditions where a boundary mode of lubrication is dominant. Samples were analyzed in an annulus-on-disk rotational test configuration to determine static and kinetic friction coefficients.

Chapter 6 extends on the results of Chapter 5 to investigate if SF constituents: HA, PRG4, and SAPL, contribute to boundary lubrication, either independently or additively, at an articular cartilage-cartilage interface. Tests were first performed in graded concentrations of SF, HA, and PRG4 alone, and a physiological concentration of SAPL. Tests were then performed in various combinations of HA, PRG4, and SAPL at physiological concentrations.

Finally, Chapter 7 summarizes the major findings of this work and discusses future directions.

1.2 Function, Composition, and Structure of Articular Cartilage

Articular cartilage is the lubricious, load bearing connective tissue at the end of long bones in synovial joints that normally facilitates low-friction and low-wear articulation [96]. During joint articulation, articular cartilage is subject to a combination of compressive, tensile, and shear forces. Compressive forces supported by cartilage in the knee can attain several times body weight [3, 72]. Tensile and shear forces within cartilage result from the frictional forces produced during joint

articulation, and additional tensile forces are associated with the curvature of the load bearing surface [11, 94]. With an individual typically taking 1-4 million steps each year, the articulation of cartilage against cartilage presents a major biomechanical challenge [93].

Adult articular cartilage is composed of relatively few chondrocytes within a fluid filled extracellular matrix. Articular cartilage is a multiphase tissue, with a fluid phase of water (68-85% of the wet weight) and electrolytes, and a solid phase comprised mostly of collagen (10-20% of the wet weight) and proteoglycans (5-10% of the wet weight) [13, 57-59, 73, 74]. The multiphasic nature of articular cartilage is responsible for its mechanical properties. Proteoglycans, with their high density of negatively charged groups, maintain tissue hydration even under high applied loads, due to their high osmotic pressure [57, 58, 60, 101]. This swelling pressure provides resistance to compression and is counteracted by the collagen network. The collagen network in turn maintains the tissue integrity of cartilage, withstanding high tensile loads generated during loading and by the swelling tendency of proteoglycans [58]. This balance between the swelling pressure of proteoglycans and the collagen network is critical to the maintenance of normal cartilage function [57].

The matrix composition and macromolecular organization of articular cartilage varies gradually with depth from the articular surface, resulting in depth-dependent mechanical properties (Fig. 1.1) [50, 84]. Articular cartilage has been divided into three zones aligned parallel to the articular surface: superficial, middle, and deep; each with distinct properties [37, 68]. In the superficial zone, chondrocytes are discoid, relatively small, and arranged in horizontal clusters at a relatively high density (~24

million cells/cm^3 in cartilage from human femoral condyle, compared to ~8 million cells/cm^3 in the middle and deep zones) [37]. These chondrocytes synthesize and secrete the putative lubricant molecule proteoglycan 4 (PRG4) [38, 43]. This specialized metabolic function distinguishes these cells from chondrocytes of the middle and deep zones [89]. The collagen network in the superficial zone, comprised of relatively small diameter fibers, is arranged in a tangential orientation relative to the articular surface, imparting a relatively high tangential tensile stiffness and strength to this zone [18, 67, 83, 103]. Conversely, proteoglycan content is relatively low, conferring a relatively low compressive stiffness [16], and allowing the opposing surfaces to distribute forces over a broad area and thereby reduce contact stress. In the middle zone, the cells are spherical, somewhat larger, and arranged in obliquely oriented clusters at a lesser density. In the deep zone, chondrocytes are oblong, even larger, and arranged in vertical columns. In the middle and deep zones, the collagen network is predominantly arranged in oblique and radial orientations respectively, imparting a relatively low tangential tensile stiffness and strength. Conversely, the proteoglycan content is relatively high in these zones, conferring a relatively high compressive stiffness.

Unfortunately, the pristine structure of the articular cartilage surface often deteriorates with aging, progressing to tissue degeneration and osteoarthritis (OA) [14]. OA is a degenerative joint disease that affects ~ 20 million Americans with a substantial economic impact of $60 billion annually [15]. OA tissue is characterized by altered structure (fissure or fibrillation [66]), composition (a low glycosaminoglycan content [10]), and function (reduced collagen integrity [95] and

softness in compression [51]). The characteristic progression of tissue degeneration in OA, from surface fibrillations, to full thickness lesions and ultimately large regions of bone-bone contact, can be quite painful and debilitating.

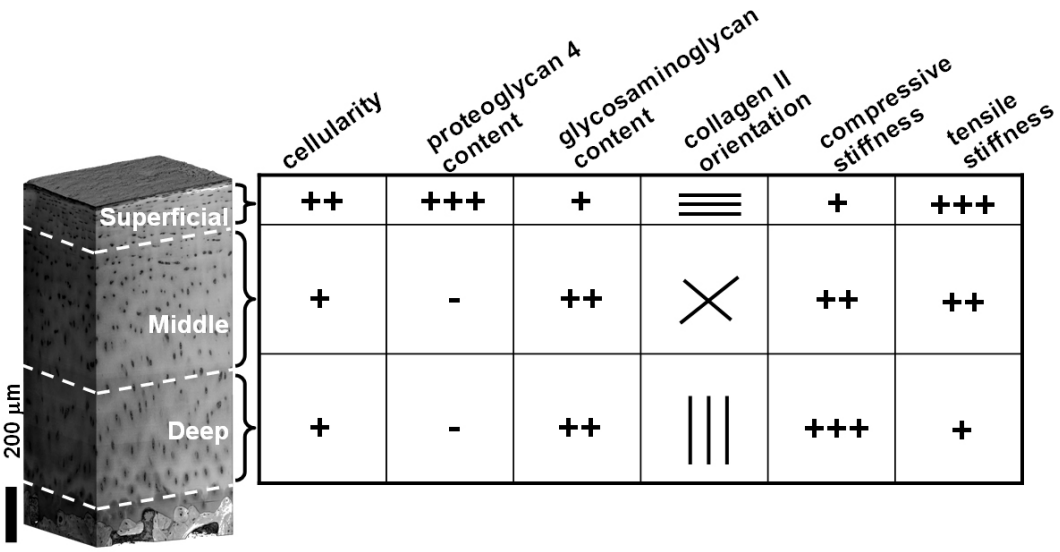

Figure 1.1: Articular cartilage structure, composition, and function are inhomogeneous and vary with depth from the superficial zone to the deep zone.

1.3 Mechanics of Articular Cartilage Lubrication

Cartilage articulates with cartilage in complex ways during the gait cycle. In the knee, the femoral surface slides and rolls against the tibial surface, maintaining contact during ~60% of the walking cycle [6]. Despite high contact stresses (1-18 MPa at heel strike [2, 8, 12, 70]), cartilage compression is relatively small, ~5-20% of the tissue thickness [4, 22, 52]. The peak translational speed between two articulating surfaces, within various joints, can range from 0.06–0.6 m/s [6]. Since joints are subject to sequential periods of rest and motion, the transition to motion represents one lubrication challenge, and steady-state motion represents an additional lubrication challenge. Indeed, the rolling, sliding, and load bearing motion of the articulating cartilage surfaces is a challenging system to test experimentally and model theoretically [6]. In general, lubricants act to reduce heat, friction and wear, and therefore play a critical role in the lifetime of most bearing systems [1].

Several theories describing the mechanisms of lubrication within synovial joints have been proposed (Fig. 1.2) [6, 104]. Factors critical to the mechanisms of lubrication are the normal and tangential forces on the articulating tissues, the relative rate of tangential motion between these surfaces, and the time history of both loading and motion [25, 26]. A number of physicochemical modes of lubrication occur in synovial joints and have been classified as fluid pressure/film or boundary [6, 104].

Several types of fluid mediated lubrication modes exist. One type is hydrostatic (Fig. 1.2A). At the onset of loading and typically for a prolonged duration, the interstitial fluid within cartilage becomes pressurized, due to the biphasic nature of

the tissue; fluid may also be forced into the asperities between articular surfaces through a weeping mechanism [63]. Pressurized interstitial fluid may therefore contribute significantly to the bearing of normal load with little resistance to shear force [6]. Also, at the onset of motion and/or loading, elastohydrodynamic (Fig. 1.2B) and squeeze film (Fig. 1.2C) types of fluid film lubrication can occur. In elastohydrodynamic lubrication, the pressure in the viscous film separating the surfaces cause significant elastic deformation of the sliding surfaces (compared to the surface roughness and film thickness). In squeeze film lubrication, the viscous lubricant is driven from between the surfaces being forced together in a normal direction. Additionally, in boosted lubrication (Fig. 1.2D), pressurized pools of trapped lubricant may contribute to the separation of cartilage surfaces as well [6, 100].

In boundary lubrication (Fig. 1.2E), load is supported by surface-to-surface contact, and the associated frictional properties are determined by lubricant surface molecules. This mode has been proposed to be important because the opposing cartilage layers make contact over ~10% of the total area, and this may be where most of the friction occurs [71]. Furthermore, with increasing loading time and dissipation of hydrostatic pressure, lubricant-coated surfaces bear an increasingly higher portion of the load relative to pressurized fluid, and consequently, this mode can become increasingly dominant [63, 69]. Boundary lubrication, in essence, mitigates stick-slip [69], and is therefore manifest as decreased resistance both to steady motion and the start-up of motion. The latter situation is relevant to load bearing articulating surfaces after prolonged compressive loading (e.g., sitting or standing *in vivo*) [75]. Typical

wear patterns of cartilage surfaces [65] also suggest that boundary lubrication of articular cartilage is critical to the protection and maintenance of the articular surface structure.

The relevant extent to which fluid pressure/film versus boundary lubrication occurs classically depends on a number of factors [69]. When lubricant film can flow between the conforming sliding surfaces, which can deform elastically, elastohydrodynamic lubrication occurs. Pressure, surface roughness, and relative sliding velocity determine when full fluid lubrication begins to break down and the lubrication enters new regimes. As velocity decreases further, lubricant films adherent to the articulating surfaces being to contribute and a mixed regime of lubrication occurs. If the velocity decreases even further and only an ultra-thin lubricant layer composed of a few molecules remain, boundary lubrication occurs. A boundary mode of lubrication is therefore indicated by a friction coefficient (μ) (ratio of the measured frictional force between 2 contacting surfaces in relative motion to the applied normal force) during steady sliding being invariant with factors that influence formation of a fluid film, such as relative sliding velocity and axial load [21]. For articular cartilage, it has been concluded boundary lubrication is certain to occur, although complemented by fluid pressurization and other mechanisms [6, 29, 70, 100].

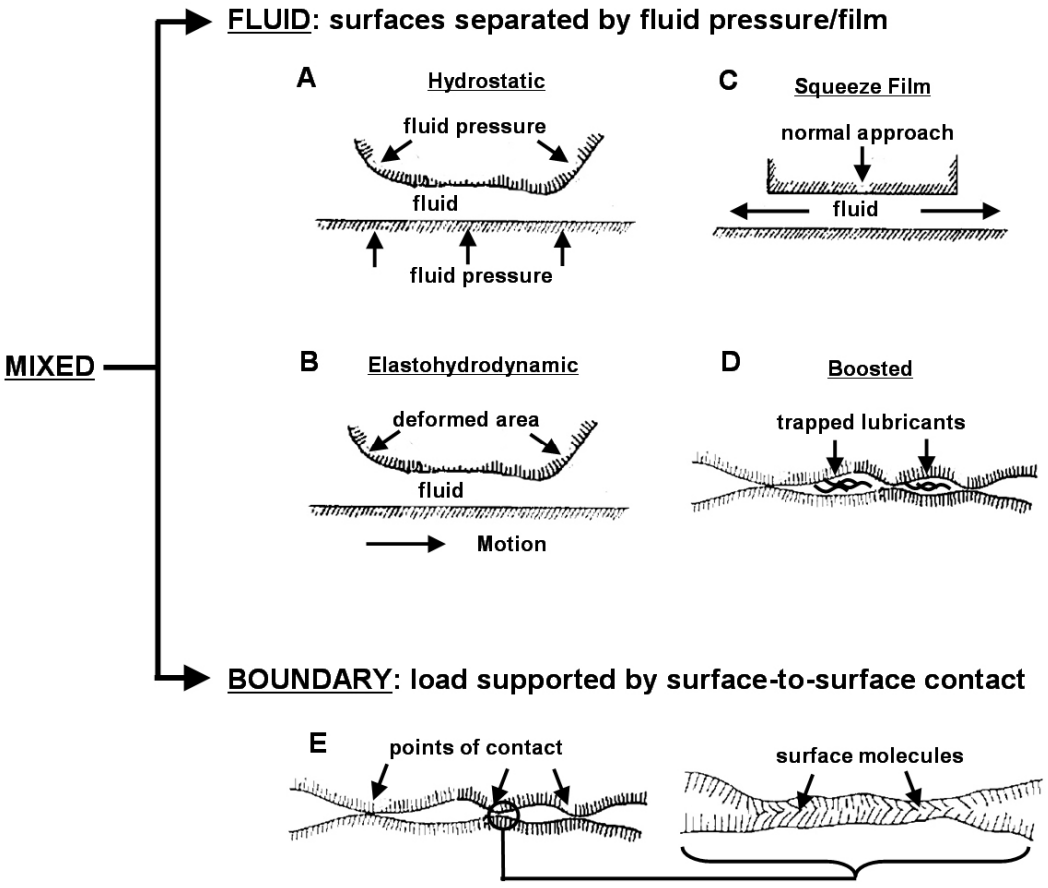

Figure 1.2: Modes of lubrication of articular cartilage (modified from [104]).

1.4 Molecular Mechanisms of Articular Cartilage Boundary Lubrication

The molecular contributors to the boundary lubrication of articular cartilage in normal mammalian joints have been subject to debate for decades. Several molecules have been implicated (Fig. 1.3). These include PRG4, SAPL, and HA. All of these molecules are present in SF [61, 85] and adsorbed to the articular surface of cartilage [76, 82, 89].

The *prg4* gene encodes for highly glycosylated proteins termed megakaryocyte stimulating factor (MSF), lubricin, and superficial zone protein (SZP) (reviewed in [40]). Lubricin was first isolated from SF and demonstrated *in vitro* lubricating ability similar to SF at a cartilage-glass interface [97]. Lubricin was later identified as a product of synovial fibroblasts [41] and also shown to possess boundary lubricating ability at a latex-glass interface by Jay *et al.* [39, 41, 43-47]. O-linked β(1-3)Gal-GalNAc oligosaccharides within a large mucin like domain of 940 amino acids [24], encoded for by exon 6, were subsequently shown to mediate, in part, this boundary lubricating ability [44]. SZP was first localized at the surface of explant cartilage from the superficial zone (Fig. 1.3.A) and isolated from conditioned medium [89]. SZP also demonstrated lubricating ability at a cartilage-glass interface [86]. These molecules will collectively be referred to as PRG4 here. PRG4 was later shown to be present at the surface of synovium, tendon [80], and meniscus [90].

The functional importance of p*rg4* was shown by mutations that cause the camptodactyly-arthropathy-coxa vara-pericarditis (CACP) disease syndrome in humans. CACP is manifest by camptodactyly, noninflammatory arthropathy, and

hypertrophic synovitis, with coxa vara deformity, pericarditis, and pleural effusion [56]. Also, in PRG4-null mice, cartilage deterioration and subsequent joint failure was observed [81]. Therefore, PRG4 expression is a necessary component of healthy synovial joints.

SAPL has long been accepted as a boundary lubricant, and was subsequently found at the surface of articular cartilage. SAPL was reported by Hills [31, 34] to exist as an oligolamellar structure (Fig. 1.3.B), reminiscent of graphite [28, 33]. SAPL are synthesized and secreted by type B synoviocytes [20, 91] and are present at the articular surface predominantly as phosphatidylcholines, dipalmitoyl phosphatidylcholine in particular [32, 36, 82].

SAPL appears to contribute to the boundary lubrication of articular cartilage; however, the supporting data is conflicting. Hills reported PRG4 isolated from SF contained SAPL, and speculated SAPL was the true lubricant with PRG4 simply acting as a carrier [92]. Hills reported digestion of SAPL with phospholipase A2 resulted in a dose-dependent inhibition of boundary lubrication at a glass-glass interface, while trypsin treatment, which would degrade PRG4, actually improved lubrication, supporting the role of SAPL in boundary lubrication [35]. Conversely, Jay *et al.* reported just the opposite at a latex-glass interface, that digestion of SF with protease free phospholipase C or A2 did not affect boundary lubrication but digesting with trypsin completely abolished boundary lubrication [42]. Indirect evidence supporting the role of SAPL exists in the form of *in vitro* and *ex vivo* studies reporting SAPL facilitated joint lubrication [77] and also inhibited cartilage degeneration in an injury model of OA when combined with HA [48, 49].

The contribution of HA to the boundary lubrication remains unclear as well. Although free HA does not bear steady load, trapped HA between or at (Fig. 1C) cartilage surfaces may contribute to a load bearing function [31, 62]. HA may also contribute indirectly to boundary lubrication due to its effect on SF viscosity [35, 54, 61, 105]. Studies using different whole joint test apparatuses where several modes of lubrication were likely operative have reported HA to be both effective [30, 55], and ineffective [49, 53, 79, 98] as a boundary lubricant. Recently, under static conditions where the intrinsic biphasic lubrication was depleted, HA was shown to function as an effective lubricant at a cartilage-cartilage interface [9]. HA has also been shown to contribute to boundary lubrication a latex-glass interface [43]. Further indirect evidence supporting the contribution of HA to boundary lubrication exists in the form *in vitro* and *ex vivo* studies demonstrating an indirect contribution to boundary lubrication (with PRG4) at a latex glass interface [46], and inhibition of experimental arthritis in combination with SAPL [49].

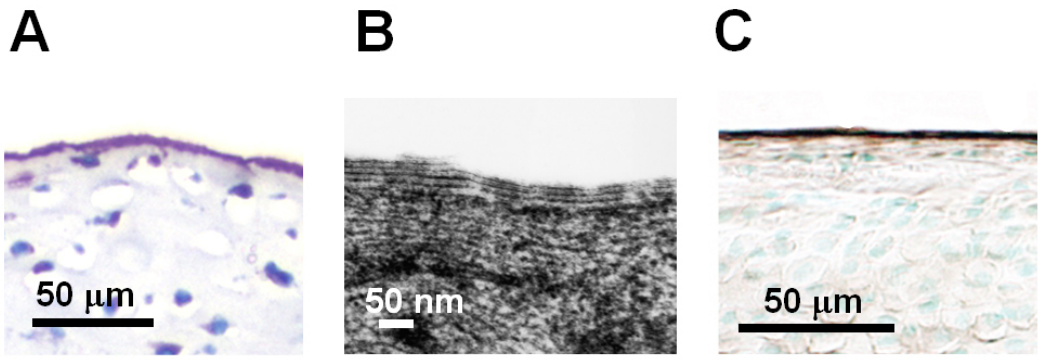

Figure 1.3: Putative articular cartilage boundary lubricants present at the articular surface. Vertical section showing (A) PRG4 in a thin layer at the articular surface and in superficial zone chondrocytes, (B) oligolamellar structure (by TEM[33]) typical of SAPL, and (C) HA in a thin layer at the articular surface [106].

1.5 Synovial Fluid Composition in Health and Disease

With injury and disease, the concentration of HA, PRG4 and SAPL in SF can be altered (Table 1.1), and this may affect the boundary lubrication of articular cartilage. The concentration of HA in human SF ranges from 1-4 mg/ml in healthy individuals [7, 17, 61, 102], and decreases after effusive joint injury [5] and in arthritic disease to ~0.1-1.3 mg/ml [19, 61]. The concentration of PRG4 in human SF ranges from 52-350 μg/ml post-mortem, and 276-762 μg/ml in SF obtained from patients undergoing arthrocentesis procedures [85]. Conversely, using a rabbit knee injury model, the concentration of PRG4 in SF decreased from 280 μg/ml to 20-100 μg/ml, 3 weeks after injury [23]. The majority of the lipids in human SF are phospholipids, whose concentration range from ~0.1-0.2 mg/ml in normal individuals, increases in osteoarthritis to ~0.2-0.3 mg/ml [61], and can decrease following traumatic injury to ~0.02-0.08 mg/ml [78]. While most phospholipids are surface active, dipalmitoyl-phosphatidylcholine (DPPC) is particularly so, and is the most abundant form present in SF at ~45% [34, 82].

The concentration of other SF constituents, including albumins and globulins (A&G) and white blood cells (WBC), can be altered in disease and injury, but do not appear to contribute to boundary lubrication [53, 99]. A&G ranges from 15-25 mg/ml in normal human SF, and increases in OA, up to 29-39 mg/ml [61], and after acute injury [64]. WBC concentration is generally $<0.2 \times 10^6$ cells/ml in normal SF, can increase up to $<2 \times 10^6$ cells/ml in OA SF, and up to 2-100 $\times 10^6$ cells/ml after acute injury [27].

Table 1.1: Synovial fluid composition in health and disease [5, 23, 27, 61, 64, 78, 85].

Constituent	SF Concentration (mg/ml)		
	Normal	OA	Acute Injury
Hyaluronan	1 - 4	0.7 - 1.1	~0.5 - 1.0
PRG4	0.05 - 0.35	0.28 - 0.76	0.02 - 0.1
Phospholipids	0.1	0.2 - 0.3	0.02 - 0.08
Albumin & Globulins	15 - 25	29 - 39	>25
Cells (10^6 WBC/ml)	<0.2	<2	2 - 100

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CHAPTER 2

SYNTHESIS OF PROTEOGLYCAN 4 BY CHONDROCYTE SUBPOPULATIONS IN CARTILAGE EXPLANTS, MONOLAYER CULTURES, AND RESURFACED CARTILAGE CULTURES

2.1 Abstract

Objective. To quantify the levels of proteoglycan 4 (PRG4) expression by subpopulations of chondrocytes from superficial, middle, and deep layers of normal bovine calf cartilage in various culture systems.

Methods. Bovine calf articular cartilage discs or isolated cells were used in 1 of 3 systems of chondrocyte culture: explant, monolayer, or transplant, for 1–9 days. PRG4 expression was quantified by enzyme-linked immunosorbent assay of spent medium and localized by immunohistochemistry at the articular surface and within chondrocytes in explants and cultured cells.

Results. Superficial chondrocytes secreted much more PRG4 than did middle and deep chondrocytes in all cultures. The pattern of PRG4 secretion into superficial culture medium varied with the duration of culture, decreasing with time in explant

culture (from ~25 μg/cm^2/day on days 0–1 to ~3 μg/cm^2/day on days 5–9), while increasing in monolayer culture (from ~1 pg/cell/day on days 0–1 to ~7 pg/cell/day on days 7–9) and tending to increase in transplant culture (reaching ~2 μg/cm^2/day by days 7–9). In all of the culture systems, inclusion of ascorbic acid stimulated PRG4 secretion, and the source of PRG4 was immunolocalized to superficial cells.

Conclusion. The results described here indicate that the phenotype of PRG4 secretion by chondrocytes in culture is generally maintained, in that PRG4 is expressed to a much greater degree by chondrocytes from the superficial zone than by those from the middle and deep zones. The marked up-regulation of PRG4 synthesis by ascorbic acid may have implications for cartilage homeostasis and prevention of osteoarthritic disease. Transplanting specialized cells that secrete PRG4 to a surface may impart functional lubrication and be generally applicable to many tissues in the body.

2.2 Introduction

Articular cartilage is the low-friction, wear resistant, load-bearing tissue at the ends of long bones in skeletal joints [44]. This tissue is composed of 3 zones, superficial, middle, and deep. Each zone has distinct properties, with matrix composition and macromolecular organization that vary gradually with depth from the articular surface. In particular, the variation in collagen network orientation and proteoglycan content confer specific and specialized mechanical and transport properties to the superficial, middle, and deep zones of cartilage [4, 20, 37]. In addition, attached to the articular surface of the superficial zone are molecules [19], including superficial zone protein (SZP) [40, 41], that appear to provide cartilage with its exquisite low-friction properties [29, 39, 47].

The proteoglycan 4 (PRG4) gene encodes for proteins that have been termed megakaryocyte stimulating factor, SZP, lubricin, and PRG4 [14]. The synthesis of PRG4 molecules plays a critical role in facilitating the low-friction properties of a variety of tissue surfaces. SZP was defined based on its synthesis and secretion from chondrocytes of the superficial zone [40, 41]. Lubricin [46, 47] was described as a metabolic product of synovial fibroblasts, abundant in synovial fluid [15], and is highly homologous to SZP [18]. In this study, we refer to these molecules with a common immunoreactivity as PRG4. A thin layer of PRG4 is present at the articular surface in healthy joints as a discrete covering over the articular surface, colocalizing with the lamina splendens [38, 41]. This presence of PRG4 at the articular surface, together with the abundance of PRG4 in synovial fluid, provides an efficient system

for boundary lubrication and low-friction articulation [16, 17]. In addition, mutations of the PRG4 gene cause dysfunction of tissues in which it is normally expressed, including pericardium and pleura, as well as cartilage [30]. In the camptodactyly-arthropathy–coxa vara–pericarditis syndrome in humans, joints exhibit camptodactyly, noninflammatory arthropathy, and hypertrophic synovitis with coxa vara deformity, the heart develops pericarditis, and the lungs demonstrate a pleural effusion. Thus, the lubrication system involving PRG4 that is present in synovial joints appears to be operative and functionally important in other tissues that require the relative sliding of their surfaces.

Explant cultures of cartilage provide a well characterized, stable in vitro system that enables the study of chondrocytes within their native extracellular matrix [12]. Chondrocytes remain viable for extended durations in explant culture, and supplementation of medium with serum stimulates proteoglycan synthesis and growth of immature cartilage [35]. The additional supplementation of medium with ascorbic acid to normal and above-normal physiologic levels [49] results in both transcriptional and posttranscriptional enhancement of collagen and aggrecan synthesis [8]. However, the extent of PRG4 secretion by cartilage explant cultures is unknown. Thus, the first objective of this study was to quantify levels of PRG4 expression by chondrocytes within explant cultures of normal bovine cartilage from the superficial, middle, and deep layers.

High- and low-density monolayer cultures of chondrocytes are also useful for a variety of purposes. In high-density monolayer culture, chondrocytes undergo relatively limited proliferation and remain phenotypically stable, as assessed by type II

collagen production, for up to 2 weeks [27]. Conversely, low-density monolayer culture offers the potential for cell expansion for therapeutic use [3], although it results in chondrocyte dedifferentiation [2]. While ascorbic acid facilitates the proliferation of chondrocytes and the formation of extracellular matrix in monolayer culture, it can also modulate the phenotype of cultured chondrocytes [9]. The limited quantitative information available on the extent of production of PRG4 is for superficial chondrocytes cultured at high density in medium supplemented with ascorbic acid and serum [22]. Thus, the second objective of this study was to quantify levels of PRG4 expression by subpopulations of released chondrocytes from the superficial, middle, and deep layers of normal bovine cartilage in monolayer culture.

Chondrocytes are used and considered for a variety of treatment modalities for damaged cartilage [36]. In the early stages of osteoarthritis (OA), the superficial zone is typically damaged and eroded [5]. To repair mild cartilage lesions before more widespread damage and OA occur, one possible treatment is the application of a cell-laden patch. The ideal function of implanted cells may include secretion of PRG4 after attachment to subsurface regions of cartilage. Thus, the third objective of this study was to quantify levels of PRG4 expression by subpopulations of released chondrocytes from the superficial, middle, and deep layers of normal bovine cartilage after transplantation onto a devitalized cartilage substrate.

2.3 Materials and Methods

Materials. Materials for tissue harvest and cell isolation and culture were obtained as previously described [7, 22].

Cartilage explant and chondrocyte isolation. Cartilage discs and chondrocytes were obtained essentially as described previously [22, 25]. Briefly, knees from 1–3-week-old and 1–2-year-old bovines with intact joint capsules were obtained from abattoirs. Under sterile conditions and after irrigation with phosphate buffered saline (PBS) supplemented with antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; all from Gibco BRL, Grand Island, NY), 8-mm–diameter osteochondral cores were harvested from the patellofemoral groove of the immature joints using the Osteochondral Autograft Transfer System (Arthrex, Naples, FL). Tissue blocks were harvested from the patellofemoral groove of the adult joints using a reciprocating saw (Johnson and Johnson, New Brunswick, NJ). These cores and blocks were fastened in a sledge microtome (Microm, Waldorf, Germany) by their underlying bone or the deep vascularized cartilage. From the adult blocks (from which the top 250 μm had been removed), 50-μm slices were obtained for use as a substrate and stored at -70°C until used. From the immature cores, superficial slices were obtained up to a depth of 250 μm (i.e., including the superficial zone), while middle and deep layers were taken at depths of 500–1,000 μm and 1,250–1,750 μm from the surface, respectively. These layers of cartilage were either punched into smaller, 3.5-mm–diameter discs or used for cell isolation. Isolated chondrocytes were obtained by

sequential enzymatic digestion (with 0.2% Pronase and 0.02% collagenase) of the cartilage slices from the 3 zones [24].

Culture of cartilage explants and chondrocytes. The cartilage discs or isolated cells were used in 1 of 3 chondrocyte culture systems. The discs were incubated as explants at 37°C in an atmosphere of 5% CO_2 with medium (low-glucose Dulbecco's modified Eagle's medium, 10 m*M* HEPES buffer, 0.1 m*M* nonessential amino acids, 0.4 m*M* L-proline, 2 m*M* L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) supplemented with 10% fetal bovine serum for up to 9 days. The medium for half of the cultures was additionally supplemented with 25 μg/ml ascorbic acid. Medium was collected every other day beginning on day 1. For all culture systems, the volume of medium used was 1 ml/10^6 initial cells/day in culture (superficial, middle, and deep explants were assumed to have 100×10^6 cells/cm^3, 40×10^6 cells/cm^3, and 40×10^6 cells/cm^3, respectively) [26]. Discs were terminated on day 9 and analyzed for DNA content to determine cell number using Hoechst 33258, assuming 7.8×10^{-12} gm DNA/chondrocyte [21].

Isolated chondrocytes were incubated as monolayers in medium for 1–9 days. Chondrocytes were plated at a high density (200,000 cells/cm^2, 400,000 cells/well) in 24-well tissue culture plates with and without additional supplementation with 25 μg/ml ascorbic acid, as well as at a medium density (50,000 cells/cm^2, 100,000 cells/well) with 25 μg/ml ascorbic acid. Beginning on day 1, selected cultures were terminated after medium collection every other day and analyzed for DNA content.

Isolated chondrocytes were also seeded onto a cartilage substrate and cultured to form an in vitro transplant model similar to that previously described [7, 23, 25]. Briefly, individual cartilage sections were secured between a polysulfone tube and an annular base, creating a well, with cartilage forming the bottom surface. Isolated chondrocytes were seeded onto the devitalized adult cartilage substrate (devoid of the superficial region) and incubated in medium for up to 9 days. Superficial, middle, or deep chondrocytes were seeded at a very high density (500,000 cells/cm^2) or a high density (200,000 cells/cm^2), both with and without additional supplementation with 25 μg/ml ascorbic acid. Medium was changed every other day, beginning on day 1. Control studies (data not shown) confirmed that the seeding efficiency of the transplanted chondrocytes onto the cartilage substrate was high (mean ± SD 88 ± 10%), with no apparent difference between chondrocyte type and seeding density.

PRG4 analysis. PRG4 expression was quantified by enzyme-linked immunosorbent assay (ELISA) of spent medium, and localized by immunohistochemistry analysis of the cultures.

Spent medium from the cultures was quantitatively analyzed in duplicate for PRG4 by indirect ELISA, as previously described [22], using mouse monoclonal antibody (mAb) 3A4 (a generous gift from Dr. Bruce Caterson, University of Wales, Cardiff, UK) [41]. Briefly, samples were diluted serially, adsorbed, and then reacted with mAb 3A4, horseradish peroxidase–conjugated secondary antibody, and ABTS substrate, with 3 washes with PBS–0.1% Tween (Bio-Rad, Hercules, CA) between each step. PRG4 levels were calculated using a PRG4 standard that was purified by

isopyknic CsCl density-gradient ultracentrifugation and ion-exchange chromatography on DEAE-Sephacel [40], verified for purity by Western blot analysis, and quantified by bicinchoninic acid protein assay (Pierce, Rockford, IL). An appropriate diluent was used so that the slopes of the control and sample absorbance curves were equivalent in the linear range of the sigmoidal curve. The assay was able to detect PRG4 to 0.01 μg/ml in 0.1 ml of medium, with a coefficient of variation of ~10% for samples run in duplicate. Preliminary experiments verified that the release of PRG4 from the surface of the explants was not just passive loss, but rather, was mostly newly synthesized material, since the amount accumulated in 1 day was an order of magnitude larger than the amount present on the surface immediately after harvest (data not shown).

PRG4 was also immunolocalized in explant, monolayer, and transplant cultures. After overnight incubation in medium supplemented with 0.1 μ*M* monensin on day 5 of culture, some cartilage samples were frozen in TissueTek OCT (Sakura USA, Torrance, CA) and sectioned normal to the articular surface, while other samples were analyzed intact to visualize the sample surface and adherent cells. Samples treated with a nonspecific mouse IgG antibody served as negative controls. The percentage of PRG4-positive cells in sample monolayer and transplant cultures was calculated (700–2,000 total cells were counted for each culture condition) using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA) and NIH Image (NIH, Bethesda, MD).

Statistical analysis. Data are presented as the mean ± SEM. All data were log-transformed to improve the uniformity of variance and were analyzed using repeated-

measures analysis of variance (ANOVA) with up to 3 repeated factors: zone (superficial, middle, and deep), culture condition (density and/or ascorbic acid), and day (1, 3, 5, 7, and 9). ANOVA statistics are reported first for each set of experiments. Planned comparisons between superficial, middle, and deep chondrocyte populations within each culture condition, as well as for superficial chondrocytes between each culture condition at each time point were conducted and *P* values are reported. Statistical analysis was implemented with Systat 9.2 (Systat, Evanston, IL).

2.4 Results

Effect of ascorbic acid on DNA content and PRG4 secretion in cartilage explants. Ascorbic acid supplementation of cultured discs increased both their chondrocyte content and the rate of PRG4 accumulation in superficial medium, which also varied with day of culture, and PRG4 was immunolocalized at the articular surface and in cells in the superficial zone of explant discs.

In the cartilage explants cultured for 9 days, cellularity varied both with the layer ($P < 0.001$) and ascorbic acid ($P < 0.05$) (Figure 2.1A). Cultured superficial explants contained more cells than the middle and deep explants in standard medium with and without ascorbic acid supplementation (each $P < 0.01$). Also, superficial explants cultured with ascorbic acid contained more cells than those without ascorbic acid ($P < 0.01$).

The average rate of PRG4 secretion by explants during culture (as assessed by accumulation in culture medium and normalized by area of articular surface) varied with layer ($P < 0.001$) and not with ascorbic acid ($P = 0.18$), but with an interaction effect between layer and ascorbic acid ($P < 0.05$) (Figure 2.1B).

The PRG4 secretion rate from superficial explants was much higher than that from middle and deep explants in both culture conditions (each $P < 0.01$). Superficial explants cultured with ascorbic acid also secreted more PRG4 (~10 μg/cm^2/day) than did those cultured without ascorbic acid (~5 μg/cm^2/day; $P < 0.05$). The PRG4 secretion rate from superficial explants into medium varied over the duration of culture ($P < 0.001$) and with ascorbic acid ($P < 0.05$), with significant interaction

effects ($P < 0.05$) (Figure 2.1C). More PRG4 accumulated in the explant culture medium with ascorbic acid compared with that without ascorbic acid, with statistically significant differences on days 3–5 and 5–7 (each $P < 0.05$) and differences approaching significance on days 1–3 and 7–9 ($P = 0.06$–0.13). PRG4 secretion decreased with culture duration from ~25 μg/cm^2/day on days 0–1 to 3 μg/cm^2/day on days 5–9.

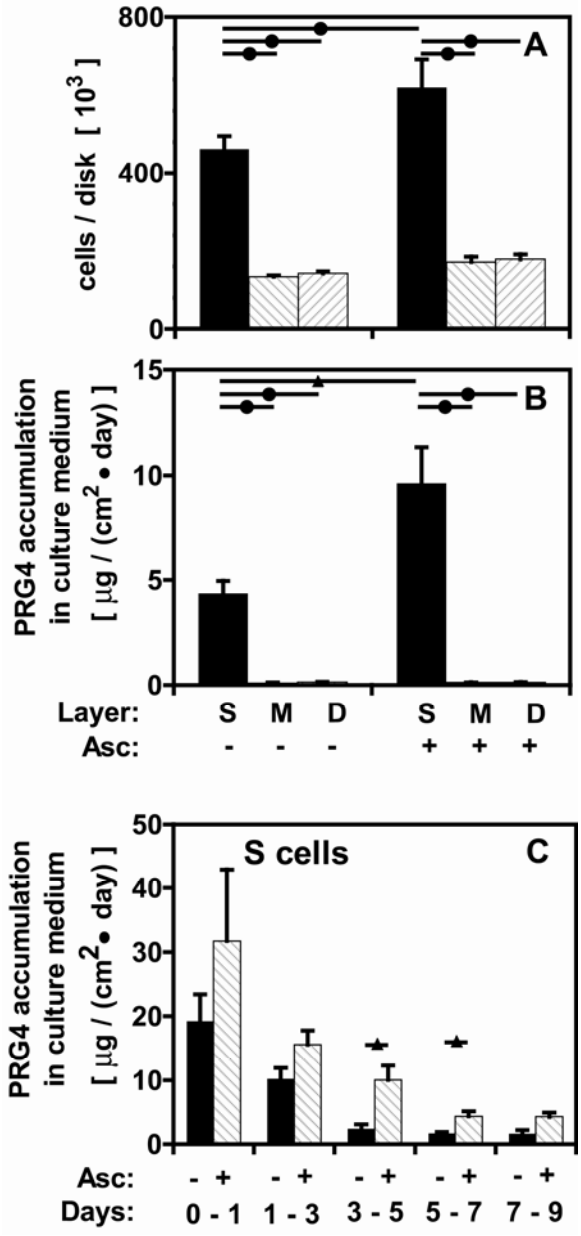

Figure 2.1: Effects of tissue region and culture condition on **A,** chondrocyte cellularity in cartilage explants, and **B** and **C,** proteoglycan 4 (PRG4) secretion rates. Superficial (S), middle (M), and deep (D) explants were cultured in medium supplemented with 10% fetal bovine serum with or without 25 μg/ml ascorbic acid (Asc) for 9 days. PRG4 accumulation in spent medium was measured by enzyme-linked immunosorbent assay. PRG4 secretion rates are shown averaged over the duration of culture for superficial, middle, and deep explants (**B**), and as a function of culture duration for superficial explants (**C**). Values are the mean and SEM of 4 experiments. ▲ = $P < 0.05$; ● = $P < 0.01$.

In freshly isolated and cultured explants, PRG4 was immunolocalized at the articular surface and in cells in the superficial zone (Figure 2.2). Positive staining was present in the day 0 sample (Figures 2.2A, E, and I) and in explants analyzed on day 5 of culture without ascorbic acid (Figures 2.2B, F, and J) as well as with ascorbic acid (Figures 2.2C, G, and K), as compared with the nonspecific IgG antibody controls (Figures 2.2D, H, and L). At higher magnification, positive cells were visualized near the surface (Figures 2.2E, F, and G), while in deeper regions, the number of PRG4 cells diminished, with none typically being visualized below 500 μm (Figures 2.2I, J, and K).

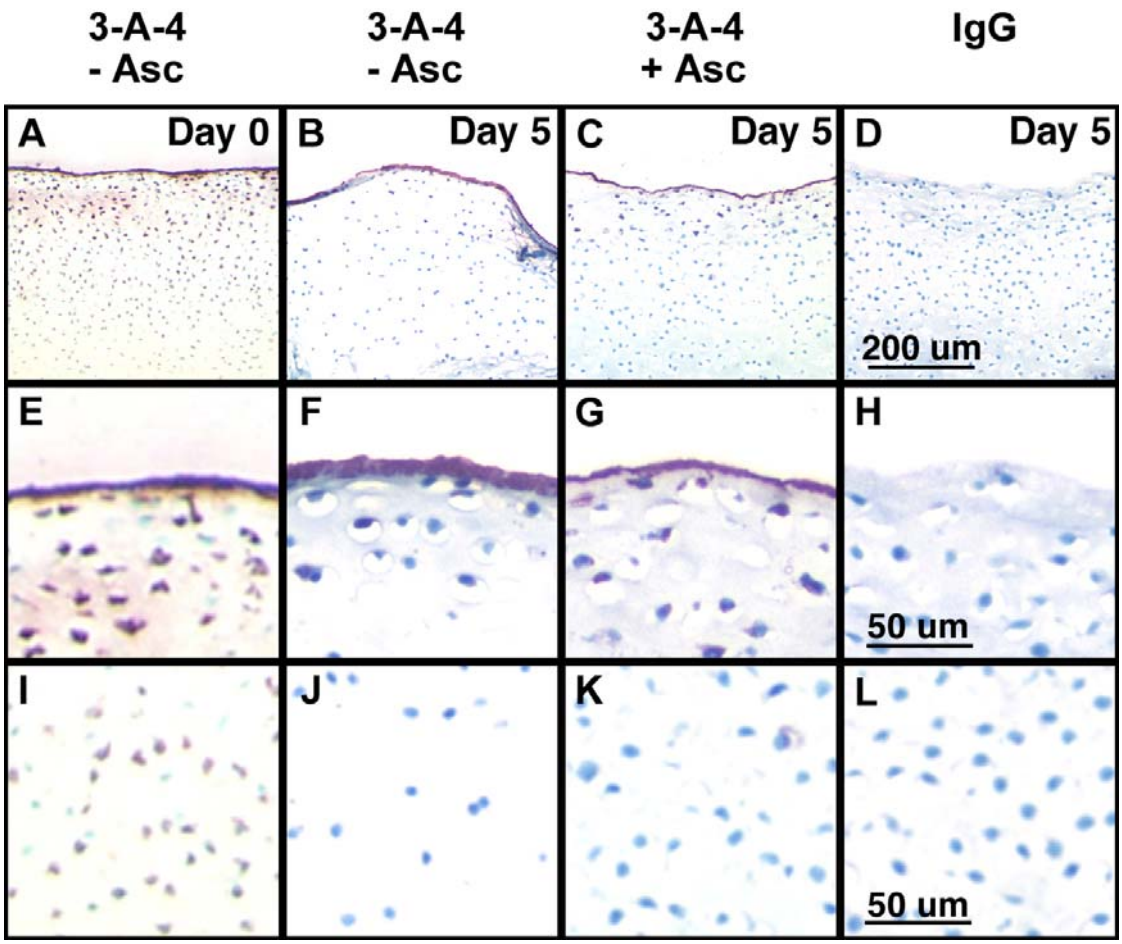

Figure 2.2: Effects of culture condition on proteoglycan 4 (PRG4) presence and expression by chondrocytes in superficial cartilage explants. PRG4 was probed with monoclonal antibody 3A4 in freshly isolated explants (**A, E,** and **I**), and in explants cultured in medium supplemented with 10% fetal bovine serum (FBS) (**B, F,** and **J**) or with 10% FBS + 25 μg/ml ascorbic acid (Asc) (**C, G,** and **K**) for 5 days. A nonspecific IgG was used as the control (**D, H,** and **L**). Magnified images of the surface (**E–H**) and deeper regions (**I–L**) are also shown.

Effect of ascorbic acid on DNA content and PRG4 secretion of chondrocytes in monolayer. As with the explants, ascorbic acid supplementation increased both the chondrocyte content of monolayer cultures and the rate of PRG4 accumulation in superficial medium. Also, PRG4 accumulation varied with the day of culture, and PRG4 was immunolocalized in cultured superficial cells.

In monolayer cultures, the number of cells after 9 days of culture varied with culture conditions ($P < 0.01$) and a culture–layer interaction ($P < 0.01$) (Figure 2.3A). Cells from each layer cultured at high density with ascorbic acid proliferated more than those cultured at high density without ascorbic acid (for superficial, $P < 0.001$; for middle and deep, $P < 0.05$ for each comparison). In medium-density cultures, the deep cells also proliferated more than the superficial cells ($P < 0.01$).

The average rate of PRG4 secretion by cells in monolayer during the culture, as assessed by accumulation in culture medium normalized to cell number, varied with layer ($P < 0.001$) and not with culture condition ($P = 0.19$), but with a layer–culture interaction ($P < 0.001$) (Figure 2.3B). More PRG4 accumulated in the superficial cell culture medium than in either the middle or the deep cell culture medium for each culture condition ($P < 0.01$ for each comparison, except high-density superficial versus middle [$P < 0.05$]). Furthermore, PRG4 accumulation in the culture medium was higher for superficial cells when cultured at high density with ascorbic acid (~3 pg/cell/day), than when cultured without ascorbic acid (~1 pg/cell/day) ($P < 0.01$). There was no difference in PRG4 accumulation rates between high- and medium-density cultures supplemented with ascorbic acid ($P = 0.82$). These results indicate

that ascorbic acid supplementation increased the average rate of PRG4 secretion by superficial chondrocytes in monolayer.

The PRG4 secretion rate by superficial cells in monolayer varied over the duration of culture ($P < 0.01$) and with culture conditions ($P < 0.01$), with a significant interaction ($P < 0.001$) (Figure 2.3C). After culture durations of >1 day, the presence of ascorbic acid in high-density cultures generally enhanced the level of PRG4 accumulation. Also, medium-density cultures supplemented with ascorbic acid eventually attained levels of PRG4 accumulation similar to those of high-density cultures with ascorbic acid. Overall, PRG4 secretion increased with the duration of culture, from ~1 pg/cell/day on days 0–1 to ~7 pg/cell/day on days 7–9.

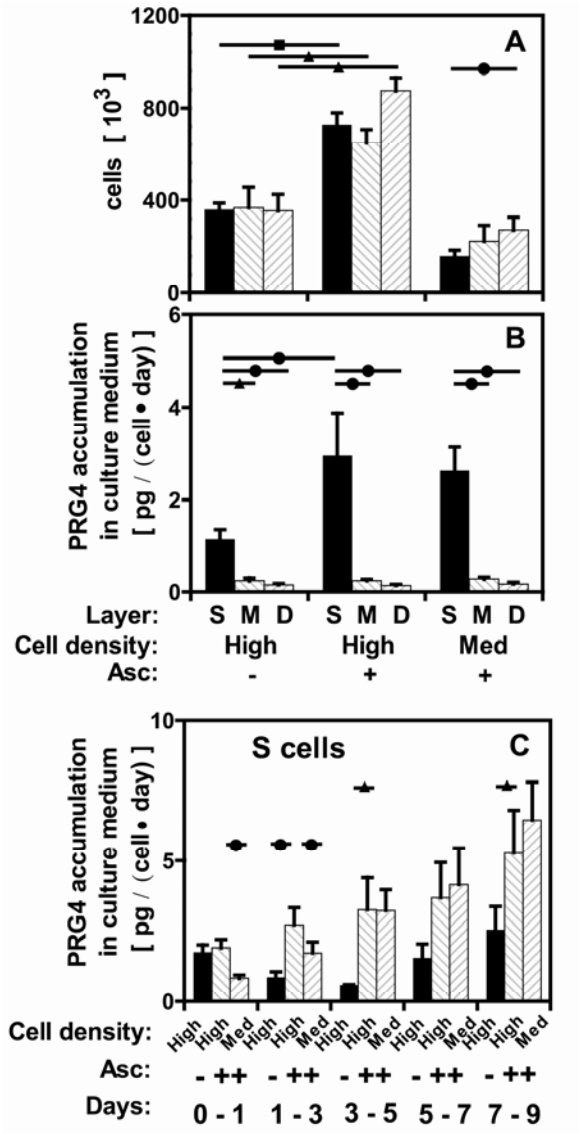

Figure 2.3: Effects of tissue region and monolayer culture condition on **A**, chondrocyte cellularity, and **B** and **C**, proteoglycan 4 (PRG4) secretion rates. Chondrocytes were isolated from superficial (S), middle (M), and deep (D) layers and then cultured as monolayers in medium supplemented with 10% fetal bovine serum. Chondrocytes were plated at high density (200,000 cells/cm^2) with or without 25 μg/ml of ascorbic acid (Asc), and at medium density (50,000 cells/cm^2) with 25 μg/ml of ascorbic acid for up to 9 days. PRG4 accumulation in spent medium was measured by enzyme-linked immunosorbent assay. PRG4 secretion rates are shown averaged over the duration of culture for superficial, middle, and deep cultures (**B**), and as a function of culture duration for superficial cultures (**C**). Values are the mean and SEM of 4 experiments. ▲ = $P < 0.05$; ▲ = $P < 0.01$; ■ = $P < 0.001$.

PRG4 was immunolocalized in superficial cell cultures (Figure 2.4) and was virtually absent from middle and deep cell cultures (results not shown) on day 5. In superficial cell cultures, PRG4 was present in the cells in high-density cultures both without (Figures 2.4A and E) and with (Figures 2.4B and F) ascorbic acid, and PRG4 was also present in cells in medium-density cultures with ascorbic acid (Figures 2.4C and G). Controls were negative for PRG4 staining (Figures 2.4D and H). In superficial cell cultures, a high percentage of cells were PRG4-positive in all culture conditions (86%, 65%, and 80% for high-density culture with and without ascorbic acid, and medium-density culture with ascorbic acid, respectively). All middle and deep cultures had almost no positive cells (0–3%).

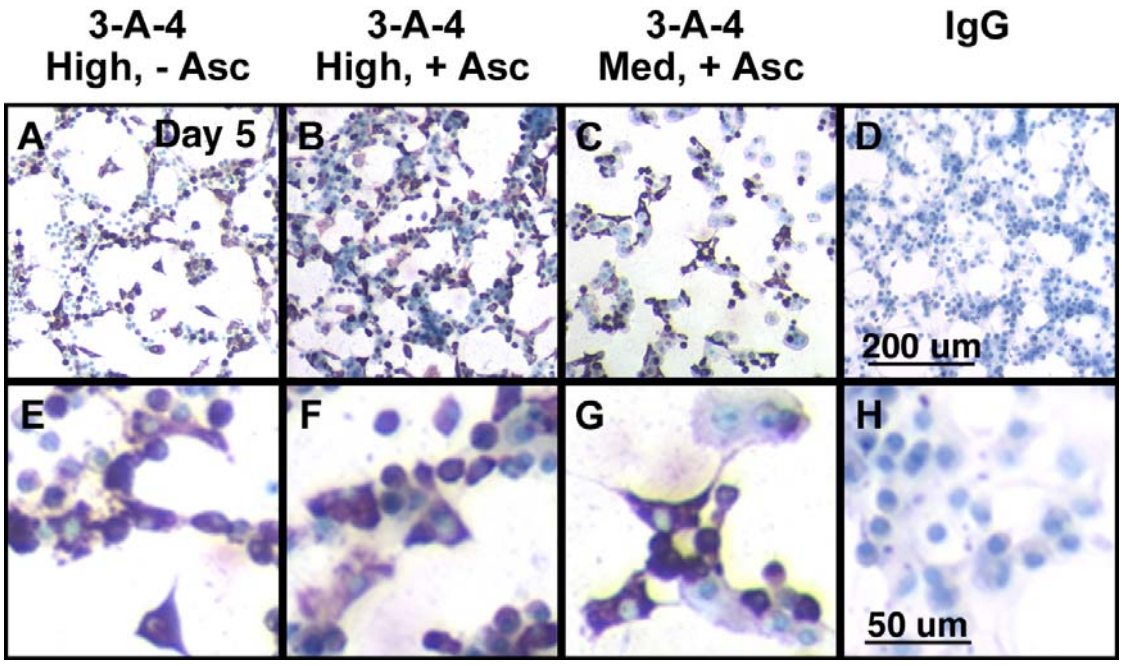

Figure 2.4: Effects of culture condition on proteoglycan 4 (PRG4) presence and expression by superficial chondrocytes in monolayer. PRG4 was probed with monoclonal antibody 3A4 in chondrocytes cultured for 5 days at high density in medium supplemented with 10% fetal bovine serum (FBS) alone (**A** and **E**) or with 25 μg/ml ascorbic acid (Asc) (**B** and **F**), and at medium density with 25 μg/ml ascorbic acid (**C** and **G**). A nonspecific IgG was used as the control (**D** and **H**). Magnified images are also shown (**E–H**).

PRG4 secretion in transplant chondrocytes. Transplanted superficial chondrocytes secreted PRG4 into the culture medium at a high rate compared with transplanted middle and deep chondrocytes. The secretion rate varied with ascorbic acid supplementation and the day of culture, and PRG4 was immunolocalized in transplanted superficial cell cultures.

In transplanted chondrocyte cultures, more PRG4 accumulated in the superficial medium compared with the middle and deep culture media (Figure 2.5A). The average rate of PRG4 secretion, as assessed by accumulation in the culture medium normalized to the cells seeded, varied between ~2–5 pg/initial cell/day in superficial cultures. The rate of PRG4 secretion by transplanted superficial chondrocytes during the culture normalized by area of cartilage varied with ascorbic acid ($P < 0.05$) and averaged ~1 μg/cm^2/day under both high- and very high–density seeding conditions.

The PRG4 secretion rate of transplanted superficial cells over the 9-day culture period varied with seeding density ($P < 0.05$) and ascorbic acid ($P < 0.05$), and exhibited significant interactions between culture duration and density ($P < 0.01$), as well as between culture duration and ascorbic acid ($P < 0.05$) (Figure 2.5B). The effect of culture duration ($P = 0.09$) approached significance. Initially, and up to day 3, more PRG4 accumulated in the culture medium of very high–density superficial cultures than in high-density superficial cultures (days 0–1 $P < 0.05$, days 1–3 $P < 0.01$). Later, more PRG4 accumulated in the medium of transplanted superficial cells with ascorbic acid than without (days 1–3 $P < 0.05$, days 7–9 $P < 0.05$). The PRG4 secretion rate seemed to increase with time, similar to that exhibited by monolayer

cultures, and eventually reached ~2 μg/cm^2/day. This rate approached the baseline secretion rate in explant cultures by the late time points of culture (Figure 2.1C).

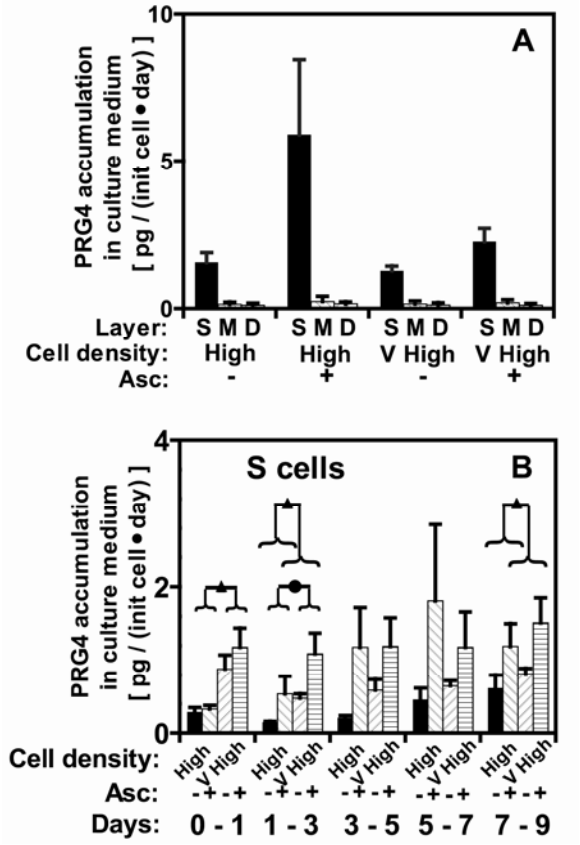

Figure 2.5: Effects of tissue region and culture condition on proteoglycan 4 (PRG4) secretion rates by chondrocytes in transplant culture. Superficial (S), middle (M), and deep (D) chondrocytes were transplanted onto devitalized adult cartilage (from which the superficial zone had been removed) and cultured in medium supplemented with 10% fetal bovine serum for 9 days. Chondrocytes were seeded at high density (200,000 cells/cm^2) and at very (V) high density (500,000 cells/cm^2), with or without 25 μg/ml ascorbic acid (Asc). PRG4 accumulation in spent medium was measured by enzyme-linked immunosorbent assay. PRG4 secretion rates are shown averaged over the duration of culture for superficial, middle, and deep cultures (**A**) and as a function of culture duration for superficial cultures (**B**). Values are the mean and SEM of 4 experiments. ▲ = $P < 0.05$; ● = $P < 0.01$.

PRG4 was immunolocalized in the transplanted superficial cells (Figure 2.6) and was virtually absent from middle and deep cells (results not shown) on day 5. In en face preparations, PRG4-positive cells were present in high-density cultures either without (Figures 2.6A and F) or with (Figures 2.6B and G) ascorbic acid, as well as in very high–density cultures either without (Figures 2.6C and H) or with (Figures 2.6D and I) ascorbic acid. Staining with the control antibody was negative (Figures 2.6E and J). In cross-sectional tissue preparations, PRG4-positive cells were present in all 4 culture conditions (Figures 2.6K, L, M, and N) relative to IgG controls (Figure 2.6O). The percentage of PRG4-positive cells in transplant superficial cultures ranged from 22% to 63% in high- and very high–density cultures, with and without ascorbic acid. Consistent with the relatively low levels of PRG4 secretion into the medium, samples with transplanted middle and deep chondrocytes had almost no positive-staining cells (0–1%).

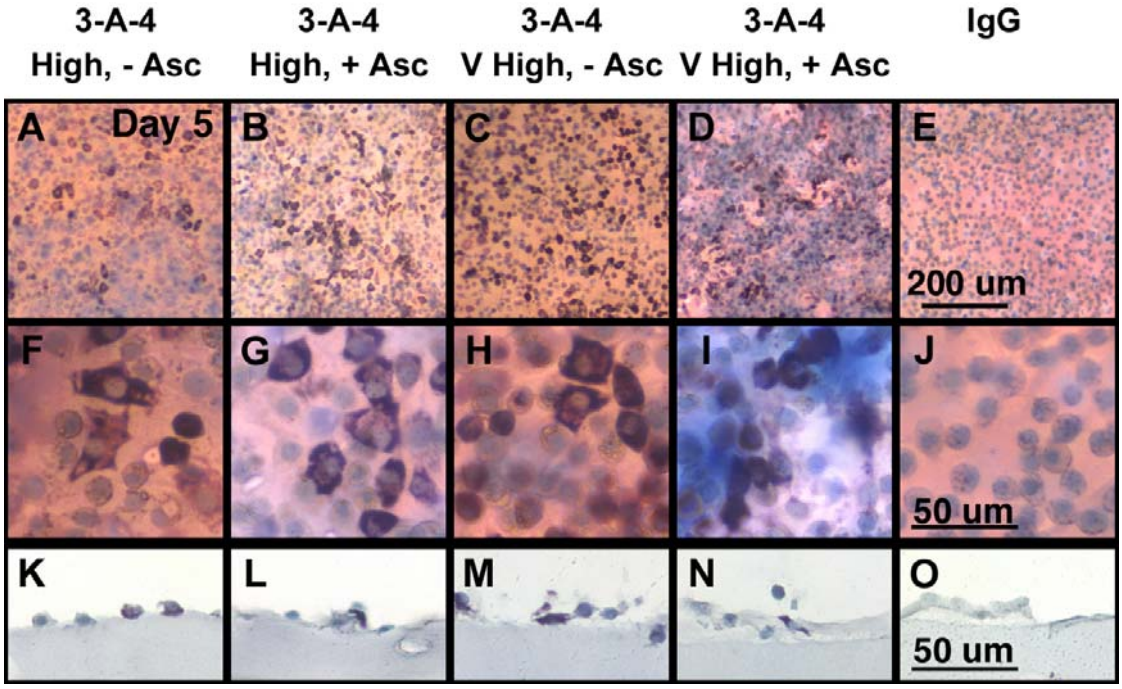

Figure 2.6: Effects of culture condition on proteoglycan 4 (PRG4) presence and expression by transplanted superficial chondrocytes. PRG4 was probed with monoclonal antibody 3A4 in transplanted chondrocytes cultured for 5 days in medium supplemented with 10% fetal bovine serum in high-density cultures alone (**A, F,** and **K**) or with 25 μg/ml ascorbic acid (Asc) (**B, G,** and **L**), and in very high–density cultures alone (**C, H,** and **M**) or with 25 μg/ml ascorbic acid (**D, I,** and **N**). A nonspecific IgG was used as the control (**E, J,** and **O**). Magnified images en face (**F–J**) and in cross section (**K–O**) are also shown.

2.5 Discussion

This study quantified and localized PRG4 expression by chondrocytes from superficial, middle, and deep layers of normal bovine calf cartilage in culture systems useful for basic investigations and possible therapies. The pattern of PRG4 secretion into the medium varied with culture duration in the various culture systems, decreasing with time in explant culture (Figure 2.1C), increasing with time in monolayer culture (Figure 2.3C), and tending to increase with time in transplant culture (Figure 2.5B). In all of the culture systems, the inclusion of ascorbic acid stimulated PRG4 secretion (Figures 2.1, 2.3, and 2.5). The source of secreted PRG4 was immunolocalized to superficial cells, whether in explants (Figure 2.2) or after release from cartilage and use in either monolayer (Figure 2.4) or transplant culture (Figure 2.6). In all of these culture systems, with serum-containing medium, the superficial chondrocyte phenotype was generally maintained with respect to PRG4 secretion.

The qualitative and quantitative PRG4 data suggest that the superficial, middle, and deep layers (defined as 0–250, 500–1,000, and 1,250–1,750 μm from the surface, respectively) of normal bovine calf cartilage contained cell populations that were either highly enriched or relatively devoid of PRG4 expression. The immunolocalization of PRG4 in explants (Figure 2.2) typically showed no PRG4-positive cells below the top 500 μm, and that in superficial monolayer culture (Figure 2.4) showed a high percentage of PRG4-positive cells compared with a very low percentage in middle and deep cultures. Furthermore, the average PRG4 secretion rate

in culture medium from the middle and deep explants, and isolated chondrocytes from the middle and deep layers, was always much lower relative to the superficial explants and chondrocytes.

The results described here extend the limited information on PRG4 secretion by chondrocytes. They indicate that the phenotype of PRG4 secretion by chondrocytes in culture is generally maintained, in that PRG4 is expressed to a much greater degree by chondrocytes from the superficial zone than by those from the middle and deep zones. We previously found that superficial cells in high-density monolayer with ascorbic acid and serum secreted 1–3 pg/initial cell/day PRG4 over 8 days in culture [22], similar to the rate obtained in this study (Figure 2.3C). Normalizing the average PRG4 secretion rate from superficial explants per cell resulted in secretion rates of ~1 pg/cell/day and 1.5 pg/cell/day with ascorbic acid supplementation, which are similar to PRG4 rates from superficial chondrocytes in monolayer culture (Figure 2.3B). Average rates of PRG4 secretion from transplanted superficial chondrocytes were similar to those in monolayer, albeit expressed in slightly different units, i.e., pg/initial cell/day versus pg/cell/day. In high- and very high–density cultures, ~2 pg/initial cell/day PRG4 accumulated in the medium, increasing significantly to ~6 and 3 pg/initial cell/day, respectively, with the inclusion of ascorbic acid in culture (Figure 2.5A). These results are self-consistent in that the same population of superficial cells appears to secrete PRG4, whether in explant, monolayer, or transplant culture.

PRG4 that accumulated in the culture medium appeared to be primarily that secreted by the chondrocytes. In vivo, a layer of PRG4 is retained at the articulating surface, bound to the lamina splendens [38, 45]. However, the amount present at the

surface of explants immediately after harvest was an order of magnitude less than that accumulated in the medium over 1 day.

A variety of mechanical and chemical stimuli may regulate chondrocyte synthesis of various molecules, including PRG4. Mechanical [11, 13] and soluble chemical stimuli, including growth factors [34] and cytokines [28], regulate chondrocyte function and cartilage matrix metabolism in general. Transforming growth factor β1 has been shown to up-regulate PRG4 synthesis at the messenger RNA level in monolayer culture [1] and protein secretion by chondrocytes in agarose culture [10]. In the present study, the physiologic cellular microenvironment was essentially retained by chondrocytes in explant culture, a well-characterized in vitro model system [12]. Conversely, chondrocytes in monolayer were isolated from their extracellular matrix and proliferated in a 2-dimensional in vitro system, as did the transplanted chondrocytes, and both were in a nonphysiologic microenvironment. The exposure of transplanted chondrocytes to a devitalized cartilage substrate may have affected PRG4 expression by the superficial cells.

The marked up-regulation of PRG4 synthesis by ascorbic acid may have implications for cartilage homeostasis and prevention of osteoarthritic disease. A high dietary intake of vitamin C has been suggested to slow the progression of OA in guinea pigs [32, 42] and humans [31]. Possible mechanisms include modulating oxidative stress, participating in immune responses, and contributing to cellular differentiation [43]. Taken together with the tribologic function of PRG4 in the joint [14, 16, 39, 46], it is possible that vitamin C slows the progression of OA due to its

up-regulation of PRG4 synthesis. How ascorbic acid up-regulates PRG4 synthesis remains to be determined.

With the advancement of sensitive, noninvasive diagnostic technology, such as magnetic resonance imaging [6, 33, 48], early detection of degradation of the superficial zone of cartilage may be possible and allow for early intervention. Current treatments do not appear to change the course of OA, with the composition, structure, and mechanical properties of the articular cartilage remaining abnormal [5]. Although the present study did not assess the necessary integration of transplanted cells with the cartilage substrate for actual resurfacing, the transplantation of a cell-laden patch with the appropriate cell phenotype may facilitate repair. Further study is required to determine the extent of cell–matrix integration and thus the potential for repair through resurfacing. Cartilage slides against cartilage, as well as other tissues, such as meniscus, synovium, and ligament. Resurfacing damaged opposed surfaces with specialized cells secreting a lubricating molecule and thus restoring functional lubrication may be generally applicable to many tissues in the body that slide against an opposed surface.

2.6 Acknowledgments

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CHAPTER 3

CHEMOMECHANICAL COUPLING IN ARTICULAR CARTILAGE: IL-1α AND TGF-β1 REGULATE CHONDROCYTE SYNTHESIS AND SECRETION OF PROTEOGLYCAN 4

3.1 Abstract

Objective. To determine if TGF-β_1 and IL-1α regulate PRG4 metabolism in explant cultures of cartilage in terms of (1) synthesis and secretion by chondrocytes in their native superficial (S) and middle (M) zones, and (2) localization at the articular cartilage surface.

Methods. Bovine calf articular cartilage disks were isolated from the S and M zones and cultured as explants for 6 days with or without serum, and with various levels of TGF-β_1 or IL-1α alone or in sequence. PRG4 expression was quantified in spent culture medium by enzyme-linked immunosorbent assay, and visualized at the articular surface and within chondrocytes by immunohistochemistry.

Results. The average rate of secretion of PRG4 was much higher for S disks than M disks, much higher in the presence of FBS, and markedly stimulated by the

presence of TGF-β_1, and inhibited by IL-1α, in a dose-dependent manner. The time course of alteration of PRG4 secretion in S disks was fairly rapid. PRG4 secretion rates were already clearly different within the first 2 days of treatment with TGF-β_1 or IL-1α and changed gradually in the subsequent days (2 to 4 and 4 to 6). The inhibitory effect of IL-1α on S disks was partially reversible. In all samples, PRG4 staining was only evident for S disks in regions at or near the articular surface, and was absent from the deeper regions of S disks and also absent from M disks. The number of chondrocytes staining positive for PRG4 generally was consistent with the observed levels of PRG4 appearing in the medium.

Conclusion. These findings demonstrate that PRG4 secretion by chondrocytes near the articular surface is highly regulated by TGF-β_1 and IL-1α in explant cultures of cartilage, and represent a step toward understanding the regulated role of PRG4 in cartilage during normal growth, homeostasis, and pathology. This marked regulation may alter the homeostatic balance of PRG4 at the surface of articular cartilage and in joints. Additionally, because PRG4 normally functions as a boundary lubricant, the inhibition of PRG4 secretion by IL-1α may contribute to the pathogenesis of arthritis.

3.2 Introduction

Articular cartilage is the low-friction, wear-resistant, load-bearing tissue at the ends of long bones in skeletal joints [26]. This tissue is composed of three zones: superficial, middle, and deep. Each zone has distinct properties, with a matrix composition and macromolecular organization that vary gradually with depth from the articular surface and confer specialized mechanical properties to these tissue regions [19]. In the superficial zone, the collagen network is arranged in a tangential orientation relative to the articular surface, imparting a relatively high tangential tensile stiffness and strength to this zone. Proteoglycan content is relatively low, conferring a relatively low compressive stiffness and allowing the opposing surfaces to distribute forces over a broad area and thereby reduce contact stress. In contrast, the collagen network in the middle and deep zones is predominantly arranged in oblique and radial orientations respectively, imparting a relatively low tangential tensile stiffness and strength. Proteoglycan content in the middle and deep zones is relatively high, conferring a relatively high compressive stiffness. Finally, attached to the articular surface of the superficial zone are molecules, including superficial zone protein (SZP) [22], that appear to provide cartilage with its exquisite low-friction properties [20].

The depth-varying metabolic activity of chondrocytes in cartilage appears to a large extent to govern the depth-varying composition, structure, and function of cartilage, and these metabolic properties, along with chondrocyte shape, size, and organization, further define the zones of articular cartilage [5]. In the superficial zone,

chondrocytes synthesize and secrete SZP, with this specialized metabolic function distinguishing these cells from chondrocytes of the middle and deep zones [22]. Chondrocytes in the superficial zone are discoid, relatively small, and arranged in horizontal clusters [23]. In the middle and deep zones, chondrocytes synthesize proteoglycan at relatively high rates. In the middle zone, the cells are spherical, somewhat larger, and arranged in obliquely oriented clusters. In the deep zone, chondrocytes are oblong, even larger, and arranged in vertical columns. Adult articular cartilage is relatively acellular [5] and transport of macromolecules within cartilage is relatively slow [16]; therefore, chondrocyte metabolism has a large influence over the pericellular and intraterritorial tissue regions near individual cells.

The proteoglycan 4 (PRG4) gene encodes for proteins that have been termed megakaryocyte-stimulating factor, SZP, lubricin, and PRG4 [6]. Lubricin, a metabolic product of synovial fibroblasts, is abundant in synovial fluid and highly homologous to SZP [8]. PRG4 molecules appear to play a critical role in facilitating low-friction properties of a variety of tissue surfaces. In this chapter, we refer to these molecules with a common immunoreactivity as PRG4. A thin layer of PRG4 is present at the articular surface in normal healthy joints as a discrete covering over the articular surface, colocalizing with the lamina splendens [22]. The presence of PRG4 at the articular surface, the abundance of PRG4 in synovial fluid, and the mechanical motion of the joint and joint loading provide an efficient system for boundary lubrication and low-friction articulation. In addition, mutations of the PRG4 gene cause dysfunction of tissues in which it is normally expressed, including the pericardium and pleura as well as cartilage [14]. In the camptodactyly-arthropathy-coxa vara-pericarditis

syndrome in humans, joints exhibit camptodactyly, noninflammatory arthropathy, and hypertrophic synovitis with coxa vara deformity; the heart is afflicted with pericarditis; and the lung demonstrates a pleural effusion. Thus, the lubrication system involving PRG4 present in synovial joints appears to be operative and functionally important in other regions that require the relative sliding of opposing tissue surfaces.

Chemical and mechanical factors are coupled in articular cartilage in a number of ways [18] (Fig. 3.1). Chondrocyte functions and cartilage matrix metabolism are regulated by imposed mechanical [4] and chemical stimuli, with the latter including growth factors and cytokines [12]. The mechanical and transport properties of cartilage determine how such mechanical and chemical stimuli present at the surfaces or boundaries of cartilage tissue are transduced into signals at the microenvironmental level. The cellular microenvironment regulates cell synthesis of various molecules—including matrix components and matrix-modifying enzymes—as well as cell fate, which may be to proliferate, apoptose, necrose, or differentiate. The extracellular microenvironment may directly affect the assembly and loss of extracellular matrix components. The resultant balance between synthesis, assembly, and loss of matrix components determines the tissue content of specific molecules. Accordingly, the accretion of the bulk of the tissue—including proteoglycan, collagen, and other matrix molecules—as well as of the surface of the tissue—including lubricating molecules—is regulated through a number of metabolic mechanisms. In turn, the quantity and structure of specific matrix molecules determine the mechanical and transport properties of cartilage. Thus, a key to understanding dynamic processes of cartilage, such as growth, maturation, homeostasis, and degeneration, is elucidating at various

levels of detail the chemomechanical coupling processes that exist in and govern articular cartilage.

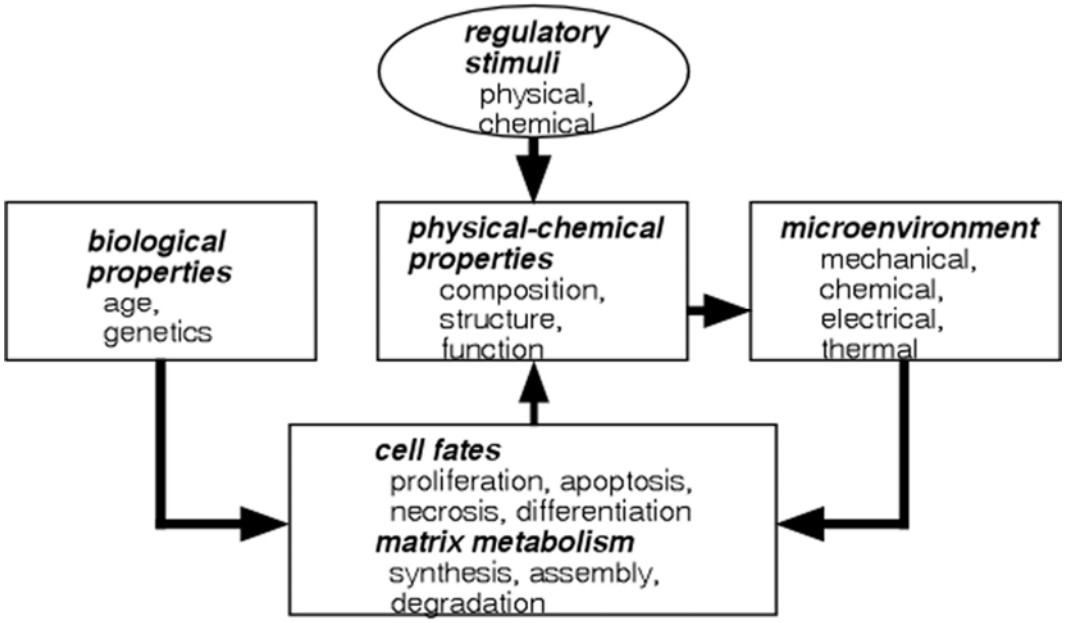

Figure 3.1: Model of cartilage dynamics. Block diagram shows the influence of environmental regulatory stimuli and biologic properties on cartilage remodeling, which may lead to cartilage growth, degeneration, or repair.

In joint injury and arthritis, transforming growth factor-beta 1 (TGF-β_1) and interleukin-1 alpha (IL-1α) are present at relatively high levels [15] and may regulate chondrocyte synthesis of PRG4 as well as turnover of PRG4 adhered to the articular surface. Cytokines in general appear to play a major role in disease states of cartilage, where an imbalance of synthesis and degradation exists. Certain cytokines, such as IL-1, exhibit net catabolic effects on chondrocytes and cartilage by both inhibiting synthesis of extracellular matrix and stimulating degradation [13]. Certain growth factors, including TGF-β [10], exhibit net anabolic effects on cartilage, stimulating synthesis of matrix and inhibiting degradation. Simulating pathologic conditions can be helpful in understanding the effects on specific functional molecules. Previous qualitative studies of PRG4 regulation by TGF-β_1 [1, 3] and IL-1α [3] have examined chondrocytes from full-thickness cartilage in terms of mRNA expression in monolayer culture [1] and protein secretion in agarose culture from chondrocyte subpopulations [3]. These studies suggest that PRG4 expression in chondrocyte cultures is inhibited by IL-1α and stimulated by TGF-β_1. However, it is unclear if these factors also regulate PRG4 in the native cartilage environment. Explant cultures maintain a number of characteristic features of cartilage, including cell-matrix interactions and biosynthetic phenotype [25].

The objective of the present study was to determine if TGF-β_1 and IL-1α regulate PRG4 metabolism in explant cultures of cartilage in terms of (1) synthesis and secretion by chondrocytes in their native superficial (S) and middle (M) zones, and (2) localization at the articular cartilage surface.

3.3 Materials and Methods

Cartilage Explant Harvest and Culture. Cartilage explants were isolated from regions of cartilage that either included or were devoid of the superficial zone, essentially as described previously [11]. Osteochondral cores were harvested from the patellofemoral groove of immature (1 to 3 weeks old) bovine knees. These cores were cut in a sledge microtome to obtain slices (0.3 mm thick) from the superficial (S, 0 to 0.3 mm) and middle (M, 0.6 to 0.9 mm) layers. These layers of cartilage were then punched into smaller 3-mm diameter disks. During harvest, cartilage was maintained hydrated by irrigation with phosphate-buffered saline supplemented with antibiotics and antimycotic (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B).

Cartilage disks were then incubated with or without serum, and with various levels of IL-1α or TGF-β_1 alone or in sequence. Disks were incubated in a basal medium (Dulbecco's modified Eagle's medium [DMEM], 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B) at 37° C in an atmosphere of 5% CO_2, supplemented with 25 μg/mL ascorbic acid, 0.01% BSA (0% FBS), or 10% FBS, as well as graded levels (0, 0.1, 1, 10 ng/mL) of either recombinant human IL-1α or porcine TGF-β_1. Every 2 days, medium (0.360 mL per S disk and 0.175 mL per M disk) was replaced, and the spent medium collected for subsequent analysis. These amounts of medium were chosen to achieve a medium volume of 1 mL per million cells per day, with S disks given more medium due to

their higher cell density [11]. Some disks were analyzed for the reversibility of effects of IL-1α by TGF-β_1 by first treating on days 0 to 6 with graded levels of IL-1α, rinsing on day 8 with basal medium over 48 hours, and then treating during days 8 to 12 with medium supplemented with 10 ng/mL of TGF-β_1.

PRG4 Secretion. PRG4 expression from cartilage disks was quantified in spent medium by indirect ELISA as described previously [9], using the monoclonal antibody (mAb) 3-A-4 (a gift from Bruce Caterson, PhD [22]). Briefly, medium samples were diluted serially, adsorbed, and then reacted with mAb 3-A-4, horseradish peroxidase-conjugated secondary antibody, and 2,2-azino-di(3-ethylbenzthiozoline-sulfonate-(6)) substrate, with three washes with PBS + 0.1% Tween between each step. PRG4 levels were calculated using purified bovine standards [22].

PRG4 Immunolocalization. The presence of PRG4 at the articular surface and within chondrocytes was determined qualitatively in freshly explanted disks and also in cultured disks that were terminated at day 6. To visualize PRG4 as a function of depth from the articular surface, samples were analyzed by immunohistochemistry using the monoclonal antibody (mAb) 3-A-4, essentially as described previously [9]. Here, samples were incubated overnight in medium supplemented with 0.1 μM monensin and then rinsed with PBS. Cryosections of a thickness of 5 μm were prepared, reacted with mAb 3-A-4, and detected with a peroxidase-based system. The stained samples were viewed to identify immunoreactive cells, indicating synthesis of PRG4, as well

as to assess the presence of adherent PRG4 at the articular surface. Results were documented by photomicroscopy. To visualize PRG4 macroscopically at the sample surfaces, disks were rinsed in PBS, fixed with 4% paraformaldehyde, reacted with mAb 3-A-4, and detected with a peroxidase-based system essentially as described above. Results were documented by digital photography. As negative controls, some samples were probed with a nonspecific isotype-matched antibody.

Statistical Analysis. Quantitative data are expressed as the mean ± SEM. Effects of cartilage layer, medium components, and culture duration on PRG4 secretion were assessed by repeated-measures ANOVA of the data after log transformation (to improve the uniformity of variance among the experimental groups).

3.4 Results

PRG4 Secretion. The average rate of secretion of PRG4 over the first 6 days of culture (Fig. 3.2A) was much higher for S disks than M disks ($P < 0.01$), much higher in the presence of FBS ($P < 0.05$), and markedly affected by the presence of TGF-β_1 or IL-1α ($P < 0.05$) at different doses ($P < 0.001$). Several interaction effects were also apparent. The dose-dependent effect depended on the tissue layer, being marked for S disks but not M disks ($P < 0.01$). In addition, the dose-dependent effects were distinct for TGF-β_1 and IL-1α, with the former causing a stimulation and the latter causing an inhibition ($P < 0.001$). Also, the effect of serum depended on the tissue layer, being marked for S disks but not M disks ($P < 0.05$). The PRG4 secretion rate by S disks cultured in medium with 10% FBS was 13 ± 3 μg/(cm^2/day) and was upregulated by TGF-β_1 and downregulated by IL-1α in a dose-dependent manner, reaching a high of 118 ± 26 μg/(cm^2/day) with 10 ng/mL TGF-β_1 and a low of 3 ± 1 μg/(cm^2/day) with 10 ng/mL IL-1α. For S disks cultured in medium without FBS, the trends in regulation of PRG4 secretion were similar, although the rates were lower, being 3 ± 1 μg/(cm^2/day) in basal medium and 62 ±18 and 2 ± 1 μg/(cm^2/day) with 10 ng/mL of TGF-β_1 and 10 ng/mL IL-1α respectively.

The time course of alteration of PRG4 secretion in S disks was fairly rapid (Fig. 3.2B). The secretion rate varied with days in culture ($P < 0.01$) as well as the presence of serum ($P < 0.05$) and cytokine ($P < 0.001$) as noted overall for the 6-day period. PRG4 secretion rates were already clearly different within the first 2 days of treatment with TGF-β_1 or IL-1α and changed gradually in the subsequent days (2 to 4

and 4 to 6). There was a significant interaction between the effect of cytokine and days in culture ($P < 0.01$), as indicated by the secretion rates of cultures treated with the maximum dose of TGF-β_1 increasing with culture duration while decreasing in cultures treated with IL-1α, and also decreasing (to a lesser extent) without any IL-1α. For S disks cultured in medium without FBS, the trends in regulation of PRG4 secretion were similar, although the rates of secretion were lower. Secretion rates from M disks (Fig. 3.2A) were only slightly above the threshold of detectability, 0.1 $\mu g/(cm^2/day)$, even when cultured with 10 ng/mL TGF-β_1.

The inhibitory effect of IL-1α on S disks was partially reversible. After IL-1α treatment for the first 6 days of culture, the secretion rate from S disks during days 8 to 12 was 2.4 ± 1.4 $\mu g/(cm^2/day)$ in basal medium with or without 10% FBS, 0.2 ± 0.2 $\mu g/(cm^2/day)$ with 10 ng/mL IL-1α, and back up to 12.9 ± 4.4 $\mu g/(cm^2/day)$ with 10 ng/mL TGF-β_1.

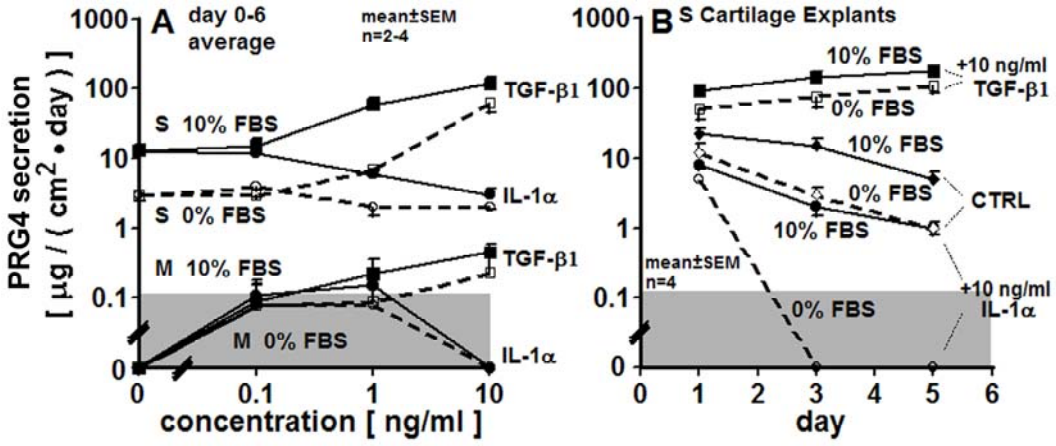

Figure 3.2: PRG4 secretion by chondrocytes in cartilage explant cultures. Articular cartilage disks (0.3 mm thick, 3 mm diameter) from the superficial (S, 0 to 0.3 mm), and middle (M, 0.6 to 0.9 mm) zones of the patellofemoral groove of immature (1 to 3 weeks old) bovine knees were incubated as explants for 6 days at 37°C in an atmosphere of 5% CO_2 with medium (DMEM +25 µg/mL ascorbic acid) with 0.01% BSA (0% FBS) or 10% FBS as well as graded levels (0, 0.1, 1, 10 ng/mL) of either recombinant human IL-1α or porcine TGF-β_1. Culture medium was changed every 2 days and collected for PRG4 analysis by indirect ELISA using the monoclonal antibody 3-A-4. **A,** Effect of recombinant human IL-1α and porcine TGF-β_1 concentration on the average rate of PRG4 secretion by explants during the culture. **B,** Effect of days in culture on the PRG4 secretion rate by S explants with 10 ng/mL of recombinant human IL-1α or porcine TGF-β_1. Shaded regions indicate levels at or below assay sensitivity.

PRG4 Immunolocalization. PRG4 was localized differentially in cartilage samples depending on the culture conditions. In all samples, PRG4 staining was only evident for S disks in regions at or near the articular surface, and was absent from the deeper regions of S disks and also absent from M disks. The number of chondrocytes staining positive for PRG4 generally was consistent with the observed levels of PRG4 appearing in the medium (Fig. 3.2). Vertical sections of freshly isolated (Fig. 3.3A) and cultured control samples (0 or 10% FBS, Fig. 3.3Ciii, iv) showed many PRG4 positive chondrocytes, as did samples treated with TGF-β_1 (Fig. 3.3Cvii, viii). In contrast, samples treated with IL-1α showed relatively few PRG4 positive chondrocytes (Fig. 3.3Cv, vi). PRG4 was immunolocalized macroscopically at the articular surface of all the intact disks, both freshly isolated (Fig. 3.3B) and cultured (Fig. 3.3D). Vertical sections of freshly isolated (Fig. 3.3A) and cultured control samples (0 or 10% FBS, Fig. 3.3Ciii, iv) showed a fairly regular staining for PRG4 at the articular surface, as did samples treated with IL-1α (Fig. 3.3Cv, vi). Samples treated with TGF-β_1 showed a variable staining for PRG4 at the articular surface (Fig. 3.3Cvii, viii). Control samples using nonspecific primary antibody were appropriately PRG4 negative.

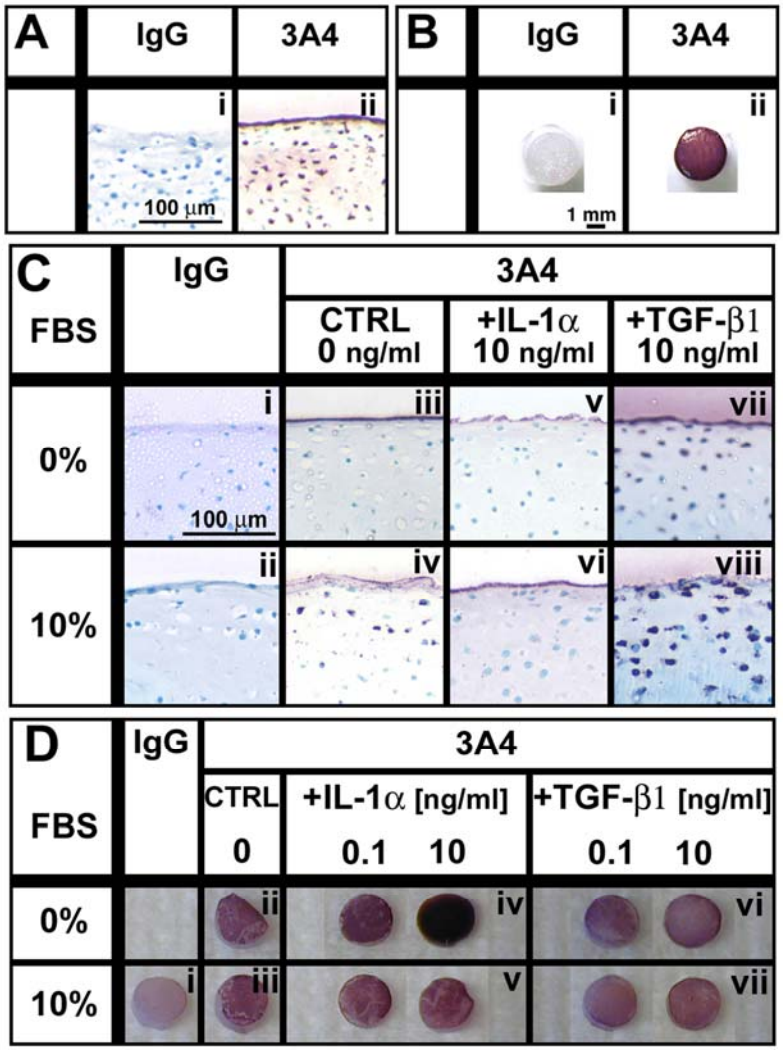

Figure 3.3: PRG4 immunolocalization in fresh and cultured superficial (S, 0 to 0.3 mm) 3-mm cartilage explants with the monoclonal antibody 3-A-4 (a nonspecific IgG antibody was used as a negative control). **A,** Vertical cryosections of fresh S disks after overnight incubation in medium with 0.1 μM monensin to identify cells synthesizing PRG4. **B,** Macroscopic en face views of the articular surface of fresh S cartilage disks. **C,** Vertical cryosections of S disks cultured as explants for 6 days at 37°C in an atmosphere of 5% CO_2 with medium (DMEM +25 μg/mL ascorbic acid) with 0.01% BSA (0% FBS) or 10% FBS as well as 0 **(iii, iv)** or 10 ng/mL of either recombinant human IL-1α **(v, vi)** or porcine TGF-β_1 **(vii, viii)** after overnight incubation in medium with 0.1 μM monensin, to identify cells synthesizing PRG4. **D,** Macroscopic en face views of the articular surface of S disks cultured as explants for 6 days at 37°C in an atmosphere of 5% CO_2 with medium (DMEM +25 μg/mL ascorbic acid) with 0.01% BSA (0% FBS) or 10% FBS as well as graded levels (0 **(ii, iii)**, 0.1, 10 ng/mL) of either recombinant human IL-1α **(iv, v)** or porcine TGF-β_1 **(vi, vii)**.

3.5 Discussion

These findings demonstrate that PRG4 secretion by chondrocytes near the articular surface is highly regulated by IL-1α and TGF-β_1 in explant cultures of cartilage. IL-1α had an inhibitory effect that was partially reversible, whereas TGF-β_1 was stimulatory, both in a dose-dependent manner (Fig. 3.2A). The pattern of secretion in different culture conditions also varied, decreasing with time in cultures supplemented with IL-1α and increasing with time in those supplemented with TGF-β_1 (Fig. 3.2B). The secretion rate also decreased in control cultures as previously described [21], although not nearly as much as in cultures treated with IL-1α (Fig. 3.2B). The trends in regulation of secretion were similar for S disks cultured in medium without FBS, although the rates were lower. PRG4 was immunolocalized to the articular surface and/or in S cells in all culture conditions, both histologically (Fig. 3.3C) and macroscopically (Fig. 3.3D). The finding that PRG4 expression is so modulated in explant cultures of cartilage represents a step toward understanding the regulated role of PRG4 in cartilage during normal growth, homeostasis, and pathology.

The interpretation of the results of the present study is affected by a number of factors. The amounts of PRG4 secreted by the explants during the first two days of culture (Fig. 3.2B) was much more than that adherent initially at the surface [21]. This indicates that the PRG4 accumulation in the medium represents molecules that predominantly were synthesized during culture. The pattern of PRG4 staining (Fig.

3.3) provides additional information about the source and location of PRG4 in cartilage explants. After treatment overnight with monensin to inhibit secretion, it is readily possible to identify cells synthesizing PRG4 at relatively high amounts in vertical immunohistochemical preparations because such cells are stained intensely. In contrast, the staining for PRG4 at the articular surface in macroscopic preparations and also in vertical sections showed graded levels of staining, which may not be directly representative of the amounts of PRG4 that are present in this location. Thus, the current studies provide information primarily about chondrocyte synthesis and secretion of PRG4.

These results agree with and extend previous qualitative studies of PRG4 regulation in chondrocytes by TGF-β_1 [1, 3] and IL-1α [3]. In those studies, full-thickness populations and subpopulations of chondrocytes were used in various culture systems, and qualitative analyses indicated that PRG4 expression was inhibited by IL-1α and upregulated by TGF-β_1. The current study quantifies the extent of regulation on chondrocytes in their native S and M zones within cartilage explants by measuring the secretion rate into culture medium. The localization of PRG4 in chondrocytes in the superficial region suggests that regulation is primarily of cells residing in this region of cartilage. The reversibility of the inhibitory effect of IL-1α after a relatively short-term treatment suggests that the effects on individual cells are not permanent.

The marked regulation in PRG4 expression may alter the homeostatic balance of PRG4 at the surface of articular cartilage and in joints. The amount of PRG4 present at articulating surfaces is most likely dependent on a balanced system of

synthesis, deposition, and removal. Sources of PRG4 include synovial fibroblasts [8] and S chondrocytes [22], and PRG4 has been localized to the surfaces of cartilage [22], meniscus [24], and tendon [17]. The synthesis and retention of PRG4 may be inhibited or altered under pathologic conditions. Variant forms of the molecule may also be present, possibly due to splice variants or proteolysis [14, 17], and mechanical stimuli may remove PRG4 from the articulating surfaces as well. It is likely that turnover of PRG4 at the surface of articular cartilage is affected by not only the synthesis rate of S chondrocytes but also the presence of PRG4 in the synovial fluid from the various other sources, all of which may be subject to regulation under pathologic conditions.

Because PRG4 normally functions as a boundary lubricant [7, 8, 20], the inhibition of PRG4 secretion by IL-1α may contribute to the pathogenesis of arthritis. The apparent presence of PRG4 at the surface, even after 6 days of IL-1α treatment, may reflect the inability of induced proteases [2] to cleave this molecule from the surface. In osteoarthritis, it is possible that the extent of mechanical removal of the surface layer is in excess of the synthesis and deposition of PRG4 at the articular surface, with the net result that the normal low-friction wear-resistant function of cartilage is diminished. Conversely, the stimulation of PRG4 secretion by TGF-β_1 may be beneficial for normal cartilage function.

Identifying and quantifying the various chemomechanical coupling processes is critical for understanding the dynamic processes of articular cartilage growth, maturation, homeostasis, and degeneration. As with the dynamic regulation of extracellular matrix components in general, the synthesis and presence of PRG4 at the

articular surface are likely to be important determinants of the low-friction and wear-resistant properties of articular cartilage. In turn, these mechanical properties may affect the way in which external stimuli, especially mechanical stimuli, affect the cellular microenvironment and ultimately cell functions, including the synthesis and presence of PRG4. Therefore, the ability to modulate the dynamic regulation of the putative lubricant PRG4, whether in homeostasis or degeneration, may ultimately be useful in prolonging the maintenance or slowing the deterioration of articular cartilage's critical mechanical functions at the end of long bones.

3.6 Acknowledgments

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CHAPTER 4

DIFFERENTIAL REGULATION OF PROTEOGLYCAN 4 METABOLISM IN CARTILAGE BY IL-1α, IGF-I, AND TGF-β1

4.1 Abstract

Objective. Proteoglycan 4 (PRG4) molecules are synthesized and secreted by cells lining the synovial cavity. PRG4 is present in synovial fluid and at the articular surface, and appears to play a fundamental role in the exquisite lubrication function of articular cartilage. In joint injury and arthritis, *in vivo* metabolism of PRG4 may be affected by the altered cytokine environment, which may be associated with the altered low-friction properties of articular cartilage. The objectives of this study were therefore to determine (1) if IL-1α, IGF-I, and TGF-β1 regulate PRG4 metabolism in cartilage from the superficial zone, in terms of chondrocyte expression, and PRG4 bound at the articular surface, and (2) if these metabolic indicators correlate with PRG4 secretion.

Methods. Bovine calf articular cartilage explants containing the superficial zone were harvested and cultured for 6 days with or without 10% FBS, along with 10 ng/ml IL-1α, 300 ng/ml IGF-I, or 10 ng/ml TGF-β1. PRG4 expression within chondrocytes of cartilage disks was visualized by immunohistochemistry. PRG4 bound to the articular surface of disks was quantified by extraction and ELISA. PRG4 secreted into culture medium was quantified by ELISA and characterized by Western Blot.

Results. PRG4 expression in chondrocytes near the articular surface was highly regulated by IL-1α (inhibitory) and TGF-β1 (stimulatory), but not IGF-I, and was strongly correlated with PRG4 secretion. The amount of PRG4 bound at the articular surface was modulated by culture with FBS, and did not correlate with PRG4 secretion.

Conclusion. These results provide insight into chondrocyte metabolism of the boundary lubricant, PRG4, under conditions relevant to normal growth, homeostasis, and pathology of articular cartilage.

4.2 Introduction

Articular cartilage is a multifunctional tissue at the ends of long bones that allows for smooth articulation within diarthrodial joints [33]. Cartilage consists of three zones (superficial, middle, and deep), each with distinct matrix structure and composition [5, 17] that impart specialized mechanical properties to these regions [11, 22]. The metabolic activity of chondrocytes of the middle and deep zones impart a high proteoglycan content to these zones, and therefore provides the load bearing function. A metabolic function specific to the chondrocytes of the superficial zone, that defines the phenotype of these cells, is the synthesis and secretion of superficial zone protein (SZP) [29]. SZP is a ~345 kDa protein present at the surface of articular cartilage [29], and in synovial fluid [23], encoded for by the proteoglycan 4 (PRG4) gene [6].

PRG4 molecules appear to play a fundamental role in the exquisite lubrication function of articular cartilage [2, 7, 9, 24, 25, 34, 35]. In addition to SZP, the PRG4 gene encodes for a highly homologous protein termed lubricin [10], a ~220 kDa product of synovial fibroblasts originally isolated from synovial fluid [8]. Mutations of the PRG4 gene can cause camptodactyly-arthropathy-coxa vara-pericarditis (CACP) disease in humans [15], which results in joint failure associated with noninflammatory synoviocyte hyperplasia and subintimal fibrosis of the synovial capsule [1]. PRG4 therefore plays an essential role in maintaining joint integrity.

In joint injury and arthritis, *in vivo* metabolism of PRG4 may be affected by the altered cytokine environment, which may be associated with the altered low-

friction properties of articular cartilage [3]. *In vitro* studies have demonstrated cytokines transforming growth factor-beta 1 (TGF-β1) and interleukin-1 alpha (IL-1α), which are present at elevated levels in joint injury and arthritis [14], up-regulate and down-regulate PRG4 protein expression, respectively, from superficial zone chondrocytes in explant culture [26], as well chondrocytes in agarose culture [4]. PRG4 expression localized immunohistochemically to chondrocytes in explants from the superficial zone appeared to be regulated in a similar manner [26]. Insulin like growth factor I (IGF-I), a potent physiological regulator of proteoglycan metabolism by chondrocytes in explant culture [13, 21], may also regulate PRG4 metabolism. The potential regulatory effect of these cytokines on the amount of PRG4 bound at the surface of articular cartilage, where it could impart boundary lubricating ability [28], is not known. Furthermore, the relationship between the amount of PRG4 bound at the articular surface, as well as PRG4 expression within chondrocytes at or near the articular surface, to PRG4 secretion from cartilage explants is also unknown. Collectively, these aspects of PRG4 metabolism may be key mediators of PRG4's lubrication function.

The objectives of this study were therefore to determine (1) if IL-1α, IGF-I, and TGF-β1 regulate PRG4 metabolism in cartilage from the superficial zone, in terms of chondrocyte expression, and PRG4 bound at the articular surface, and (2) if these metabolic indicators correlate with PRG4 secretion.

4.3 Materials and Methods

Materials. Materials for tissue harvest and culture were obtained as described previously [26, 27]. In addition to the recombinant human IL-1α and porcine TGF-β1, recombinant human IGF-I was also obtained from Sigma (R&D Systems, Inc. Minneapolis, MN).

Cartilage Explant Harvest & Culture. Cartilage explants were harvested and cultured as described previously [26]. Briefly, osteochondral cores were harvested from the patellofemoral groove of immature (1-3 wk old) bovine stifle joints. These cores were cut in a sledge microtome to obtain slices (0.3 mm thick) from the superficial zone, with the articular surface intact. These layers of cartilage were then punched into smaller 3 mm diameter disks. Disks were then incubated for 6 days in a basal medium (Dulbecco's modified Eagle medium [DMEM], 10 m*M* HEPES buffer, 0.1 m*M* nonessential amino acids, 0.4 m*M* L-proline, 2 m*M* L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) at 37° C in an atmosphere of 5% CO_2, supplemented with 25 μg/ml ascorbic acid, 0.01% BSA (-FBS) or 10% FBS (+FBS), as well as 10 ng/ml IL-1α, 300 ng/ml IGF-I, or 10 ng/ml TGF-β1. Every two days, medium (0.360 ml) was replaced, and the spent medium collected for subsequent analysis.

PRG4 Analysis in Cartilage Explants.

PRG4 Immunolocalization. PRG4 expression within chondrocytes of cartilage disks was visualized by immunohistochemistry using the monoclonal antibody (mAb) 3A4, (a gift from Dr. Bruce Caterson [30]), as described previously [26]. Briefly, after 6 days of culture, disks were incubated overnight in medium supplemented with 0.1 μM monensin, and then rinsed with PBS. 5 μm thick cryosections were prepared, reacted with mAb 3A4, and detected with a peroxidase-based system. As negative controls, some samples were probed with a non-specific isotype-matched (IgG) antibody. The stained samples were viewed and documented by photomicroscopy to identify immunoreactive cells, indicating synthesis of PRG4.

The depth-associated variation in chondrocyte PRG4 expression was determined from these micrographs, essentially as described previously [18]. Briefly, a representative 740 μm wide x 300 μm deep region of each section was analyzed using a custom program written in Matlab 6.5 software (The Mathworks, Inc.). Chondrocytes expressing PRG4 (PRG4+) were identified manually. Then, the total number of PRG4+ cells, and the number of PRG4+ cells as a function of depth from the articular surface were counted. Results are presented as the number of PRG4+ cells in successive 50 μm bins below the articular surface. The total number of cells counted in each section was 300±98 (mean±SD).

PRG4 Surface Concentration. PRG4 bound to the articular surface of freshly isolated disks, and those cultured for 6 days, was determined by extraction and ELISA, as described previously [31]. Briefly, 4 3-mm disks were nutated in 0.4 ml of 4 M GuHCl, 0.02 M Tris, pH 8.2 containing protease inhibitors (0.005 M benzamidine HCl, 0.01 M N-ethylmaleimide, 0.005 M disodium EDTA, and 0.001 M

phenylmethylsulfonylfluoride) for 24 hr. A pre-cut nitrocellulose membrane was wet with 20% methanol, rinsed with PBS, and then placed in a Bio-Dot apparatus. 200 µl of samples or purified bovine standards [29] were then applied to the membrane, blocked with 5% non-fat dry milk, incubated with mAb 3A4, and then with an anti-mouse antibody conjugated to horseradish peroxidase, with rinses in PBS between each step. The sample areas were removed and reacted with ABTS substrate. Amounts of PRG4 are expressed as $\mu g/cm^2$ area of articular surface (since PRG4 has been immunolocalized at or near the articular surface of intact disks, both freshly isolated and cultured [26]). Control studies indicated medium components in standards, and the extracted cartilage components in samples, caused little interference in the PRG4 ELISA.

PRG4 Analysis in Spent Culture Medium.

PRG4 Secretion. PRG4 secreted into culture medium was quantified by ELISA using mAb 3A4, as described previously [27]. Briefly, medium samples pooled from the 6 day culture were diluted serially, adsorbed, and then reacted with mAb 3A4, horseradish peroxidase-conjugated secondary antibody, and ABTS substrate, with three washes with PBS + 0.1% Tween between each step. PRG4 levels were calculated using purified bovine standards [30].

Western Blot. PRG4 in spent culture medium, pooled from the 6 day culture, was characterized by Western Blot, essentially as described previously [18]. PRG4 was purified from equal volumes of pooled samples by anion exchange chromatography with DEAE Sepharose™ gel, collecting the 0.3-0.6M NaCl eluate,

and then concentration with a Centricon® Plus 100 kDa MW cutoff filter. Samples containing similar proteins loads for each condition (~0.5 - 1 μg of total PRG4 per lane, as determined by ELISA with mAb 3A4) were separated by SDS-PAGE on a 4-20% gradient polyacrylamide gel, transferred to a PVDF membrane, and probed with mAb 3A4, with ECL-Plus detection. The membrane was digitized with a STORM 840 Imaging System (Molecular Dynamics, Fairfield, CT).

Statistical Analysis. Data are expressed as the mean±SEM. Effects of medium components (FBS and cytokines) on PRG4 expression by chondrocytes were assessed by ANOVA with depth as a repeated measure; a square root transformation was applied to improve the uniformity of variance amongst the experimental groups. Effects of medium components on PRG4 surface concentration were assessed by ANOVA, with Tukey post hoc tests for comparisons between experimental groups. The effect of culture on PRG4 concentration was also assessed by ANOVA with Dunnett's post hoc tests for comparisons to freshly isolated disks. Effects of medium components on PRG4 secretion were assessed by ANOVA, after a log transformation to improve uniformity of variance, with Tukey post hoc tests. The relationships between the total number of PRG4+ cells and PRG4 secretion, and PRG4 surface concentration and PRG4 secretion, were analyzed by linear regression.

4.4 Results

PRG4 Analysis in Cartilage Explants.

PRG4 Immunolocalization. PRG4 was localized differentially in cartilage disks, depending on the culture conditions (Fig. 4.1). In vertical sections from all groups, PRG4 staining was evident at the articular surface, and within chondrocytes near the articular surface. Control samples using the non-specific IgG primary antibody were appropriately PRG4 negative.

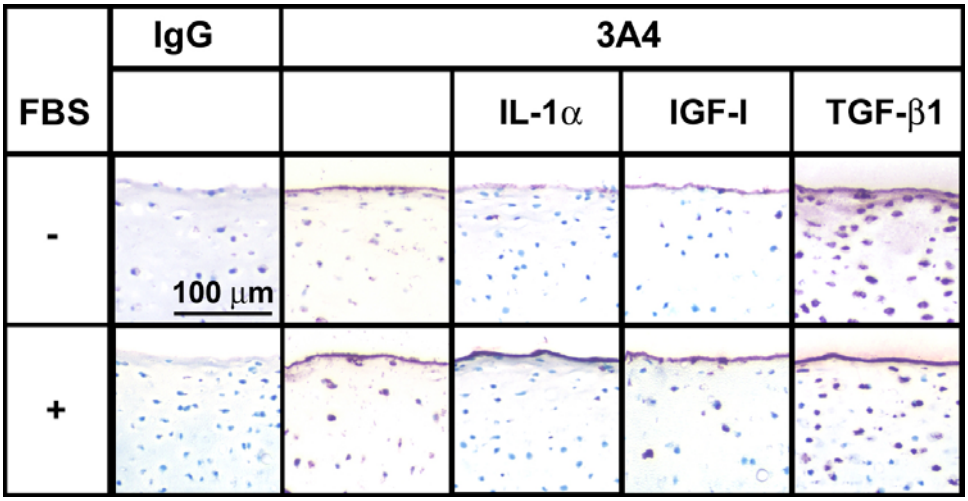

Figure 4.1: Effect of culture conditions on PRG4 presence and expression by chondrocytes in cartilage explants from the superficial zone. Disks were cultured in medium supplemented with 25 μg/ml ascorbic acid, ± 10% fetal bovine serum (FBS), as well as 10 ng/ml IL-1α, 300 ng/ml IGF-I, or 10 ng/ml TGF-β1. PRG4 was probed with mAb 3A4 in disks after 6 days of culture. A non-specific IgG was used for the control.

The total number of PRG4+ cells and depth-associated variation in chondrocyte expression was modulated by culture condition (Fig. 4.2). The total number of PRG4+ cells varied with cytokine treatment ($p<0.001$), but not with FBS ($p=0.78$) or an interaction ($p=0.61$). Untreated samples cultured with and without FBS had 51±10 PRG4+ cells, which was similar to the number of PRG4+ cells in samples treated with IGF-I 41±8 ($p=0.74$). Samples treated with TGF-β1 and IL-1α had significantly more, 228±20 ($p<0.001$), and less, 6±3 ($p<0.001$), PRG4+ cells than untreated samples, respectively. The number of PRG4+ cells also varied with depth from the articular surface ($p<0.001$), with significant depth–FBS and depth–cytokine interactions (both $p<0.001$). Untreated samples had many PRG4+ cells in the top 200 μm and very few below 200 μm, in medium -FBS (Fig. 4.2A) and +FBS (Fig. 4.2B). Samples treated with IL-1α, IGF-I, TGF-β1 appeared to have less, similar, and more PRG4+ cells, respectively, in the top 200 μm of tissue compared to untreated samples. Additionally, samples treated with TGF-β1 had PRG4+ cells below a depth of 200 μm, whereas all other groups did not.

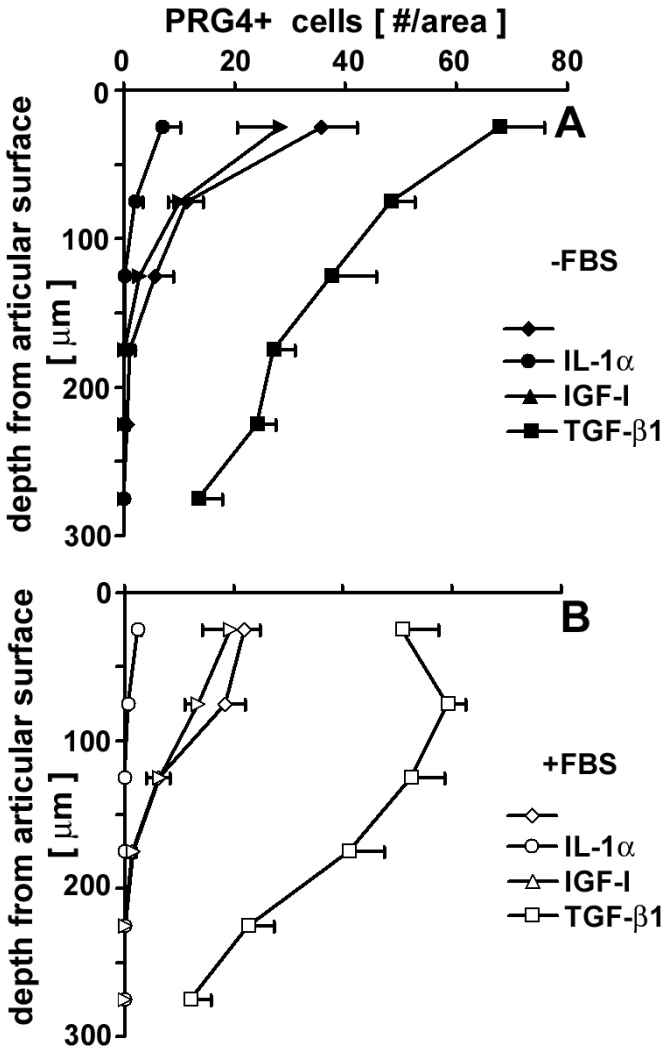

Figure 4.2: Effect of culture conditions on depth-dependent PRG4 expression by chondrocytes in cartilage explants from the superficial zone, represented as cells per area. PRG4 was probed with mAb 3A4 in disks after 6 days of culture. Data are mean ± SEM (n=6-7).

PRG4 Surface Concentration. The amount of PRG4 bound to the articular surface of disks at the end of the culture period varied with FBS ($p<0.01$), but not with cytokine ($p=0.92$) or an interaction effect ($p=0.08$) (Fig. 4.3). Untreated samples cultured in medium -FBS had 0.72±0.05 μg/cm^2 PRG4, which was similar to the amount bound to samples treated with IL-1α, 0.49±0.07 μg/cm^2 ($p=0.51$), IGF-I, 0.65±0.11 μg/cm^2 ($p=0.99$), and TGF-β1, 0.52±0.08 μg/cm^2 ($p=0.66$). Untreated samples cultured in medium –FBS did however have significantly more PRG4 bound than those cultured in medium +FBS, 0.33±0.12 μg/cm^2 ($p<0.05$). Samples cultured in medium +FBS and treated with IL-1α, 0.46±0.03 μg/cm^2, IGF-I, 0.32±0.10 μg/cm^2, and TGF-β1, 0.45±0.05 μg/cm^2, had similar amounts of bound PRG4 compared to untreated samples cultured in medium +FBS ($p=0.96 – 1$). The amount of PRG4 bound to the articular surface of freshly isolated samples (Day 0), 0.85±0.08 μg/cm^2, was significantly more than the amount bound to the surface of samples cultured in medium -FBS and treated with IL-1α or TGF-β1 ($p<0.05$), and all samples cultured in medium +FBS ($p<0.001 – 0.05$).

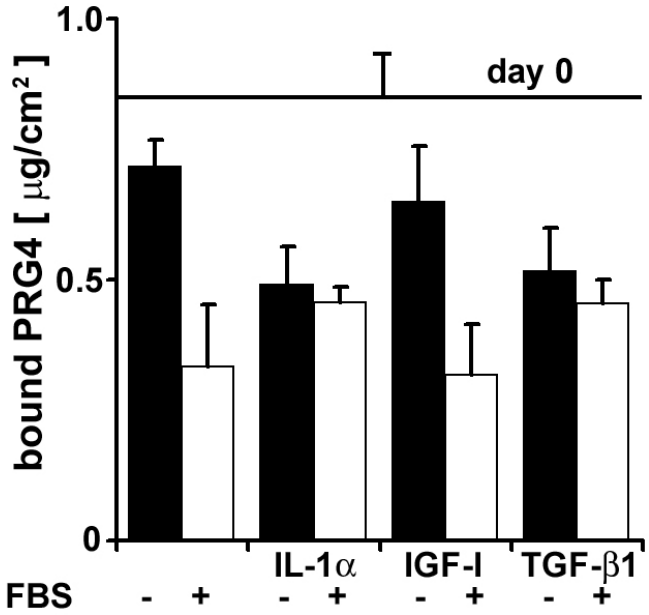

Figure 4.3: Effect of culture conditions on the amount of PRG4 bound to the surface of cartilage explants from the superficial zone after 6 days of culture. Data are mean ± SEM (n=5).

PRG4 Analysis in Spent Culture Medium

PRG4 Secretion. The average rate of PRG4 secretion over the 6 days of culture varied with FBS ($p<0.01$) and cytokine treatment ($p<0.001$), without an interaction effect ($p=0.60$) (Fig. 4.4). Samples cultured in medium -FBS secreted 1.3±0.3 μg/cm^2/day, which was similar to the amount secreted by samples treated with IGF-I, 1.9±0.7 μg/cm^2/day ($p=1.0$). Samples treated with TGF-β1 and IL-1α secreted significantly more, 65±18 μg/cm^2/day ($p<0.01$), and less, 0.05±0.04 μg/cm^2/day ($p<0.001$), PRG4 compared to untreated samples, respectively. For samples cultured in medium +FBS, the trends in regulation of PRG4 secretion were similar, although the rates were significantly higher. Untreated samples secreted 4.3±1.3 μg/cm^2/day, while those treated with IL-1α, IGF-I, TGF-β1 secreted 0.21±0.05 μg/cm^2/day, 4.8±0.8 μg/cm^2/day, and 124±15 μg/cm^2/day, respectively.

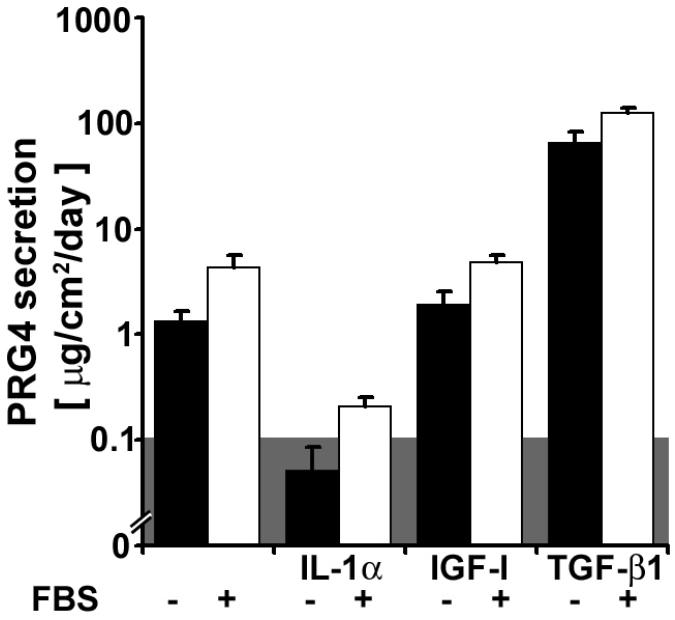

Figure 4.4: Effect of culture conditions on PRG4 secretion by chondrocytes in cartilage explants from the superficial zone. PRG4 secretion rates are shown averaged over 6 days of culture. Shaded regions indicate levels at or below assay sensitivity. Data are mean ± SEM (n=8).

PRG4 Western Blot. A major immunoreactive band with an approximate molecular weight of ~345 kDa was visualized in all samples (Fig. 4.5).

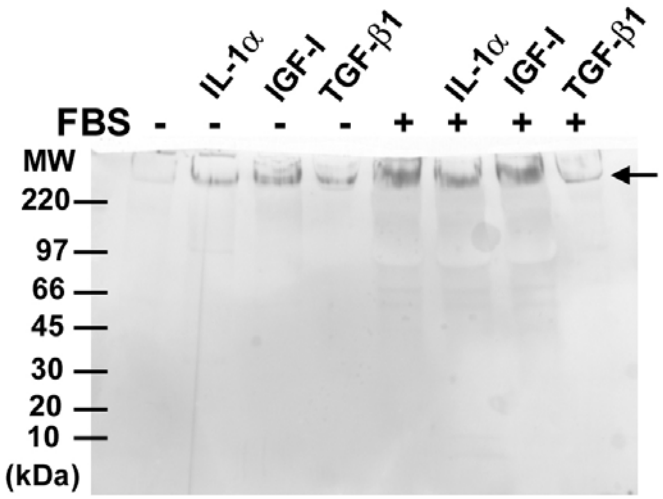

Figure 4.5: Western blot of PRG4 secreted by chondrocytes in cartilage explants from the superficial zone under various culture conditions. 0.5-1 μg PRG4 per lane (as determined by ELISA with mAb 3A4) was probed with mAb 3A4 after anion separation on a 4-20% gradient polyacrylamide gel.

PGR4 Expression Correlations. The average rate of PRG4 secretion over the 6 day culture was correlated with the total number of PRG4+ cells and the PRG4 bound to the articular surface at the end of the culture period (Fig 4.6). The average rate of PRG4 secretion showed a strong correlation with the total number of PRG4+ cells ($p<0.001$) (Fig. 4.6A), with a correlation coefficient, $R^2 = 0.61$. Conversely, PRG4 secretion did not correlate with the amount of PRG4 bound to the articular surface ($p=0.75$) (Fig. 4.6B).

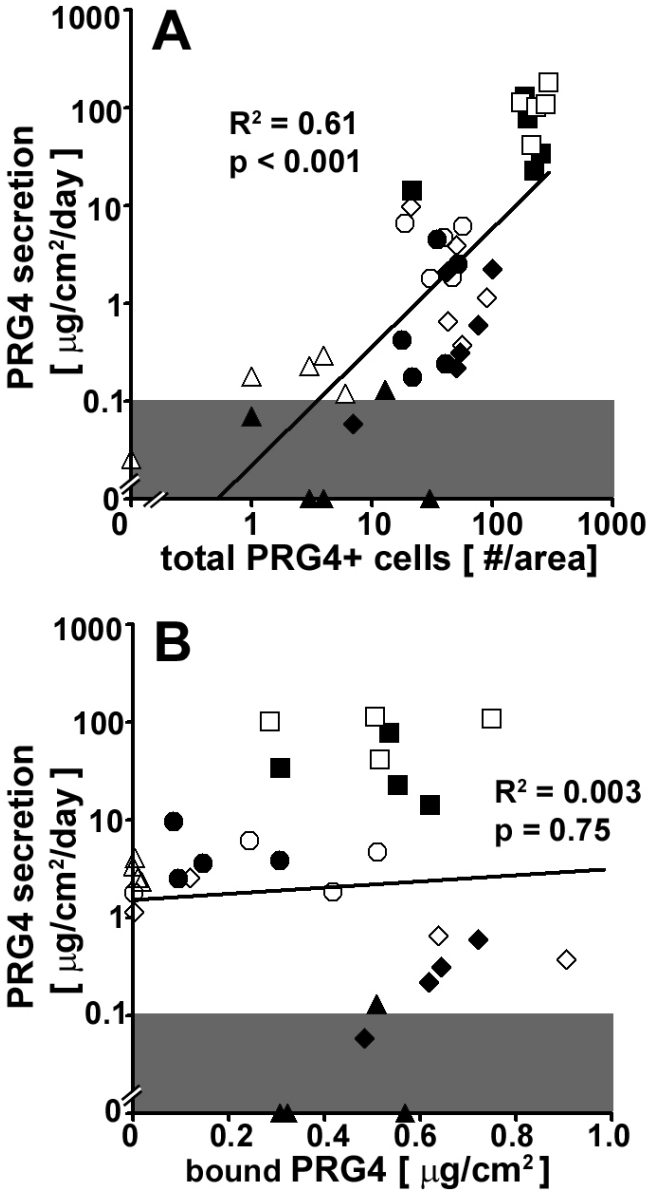

Figure 4.6: Correlation of PRG4 secretion by chondrocytes in cartilage explants from the superficial zone over 6 days of culture to (**A**) the total PRG4+ cells per area in the cartilage explants, and (**B**) the amount of PRG4 bound at the surface of the cartilage explants, at the end of the culture duration. Shaded regions indicate levels at or below assay sensitivity

4.5 Discussion

These results indicate that (1) PRG4 expression in chondrocytes near the articular surface is highly regulated by IL-1α and TGF-β1, and is strongly correlated with PRG4 secretion; (2) PRG4 bound at the articular surface is modulated by culture with FBS, and does not correlate with PRG4 secretion. At 10 ng/ml, IL-1α had an inhibitory effect on PRG4 expression by chondrocytes, TGF-β1 had a stimulatory effect, whereas 300 ng/ml of IGF-I had no detectable effect (Figs. 4.1, 4.2). The amount of PRG4 bound to the surface of cultured disks was not significantly altered by cytokine supplementation. However, culture in medium +FBS did significantly reduce the amount of surface bound PRG4 compared to freshly isolated disks (Fig. 4.3). In all culture conditions, the PRG4 secreted into medium (Fig. 4.4) over the culture duration was predominantly of the ~345 kDa form (Fig. 4.5). PRG4 secretion correlated strongly with PRG4 expression but not the amount of PRG4 bound at the articular surface (Fig. 4.6). These results provide insight into chondrocyte metabolism of the boundary lubricant, PRG4, under conditions relevant to normal growth, homeostasis, and pathology of articular cartilage.

PRG4 metabolism was assessed quantitatively in terms of chondrocyte expression, the amount bound at the articular surface, and the amount present in conditioned culture medium. The accumulation of PRG4 molecules in culture medium represents molecules predominantly synthesized, since the amount present at the articular surface before and after culture was much less, as described previously [18, 26, 27]. The reported secretion levels could be underestimations of the total synthesis,

under certain conditions where secretion rates are relatively low, depending on the as of yet unknown partition coefficient [16] of PRG4 in cartilage.

These results agree with and extend previous studies that examined the biochemical regulation of PRG4 metabolism. In a previous study [26], the authors of the present study demonstrated a dose-dependent stimulatory and inhibitory effect, of TGF-β1 and IL-1α, respectively, on PRG4 secretion by chondrocytes in explants from the superficial zone. Similar trends in PRG4 expression by chondrocytes near the articular surface were also observed, with variable staining for PRG4 at the articular surface. In the present study, the effects of TGF-β1 and IL-1α, at 10 ng/ml, on PRG4 secretion were confirmed, IGF-I was found to have no detectable effect, and the PRG4 secreted into medium was characterized as a ~345 kDa form. The previous qualitative observations regarding PRG4 expression by chondrocytes and PRG4 at the articular surface were confirmed and extended by quantitative means as well, allowing for correlative analysis with PRG4 secretion data. The lack of effect by IGF-I on PRG4 expression reported here, whose stimulatory effect on proteoglycan synthesis [21] was verified (data not shown), is different than a previously reported stimulatory effect on PRG4 protein expression by chondrocytes in agarose culture [4]. This difference may be attributable to the different culture systems used [32]. Other studies have also reported the inclusion of ascorbic acid in culture media [27], and mechanical stimuli in the form of dynamic shear, up-regulates PRG4 expression by chondrocytes in explants. Collectively, these results support the hypothesis that chemo-mechanical processes at the local microenvironment govern chondrocyte metabolism [20], specifically of PRG4 [26], within articular cartilage.

The accumulation of PRG4 within synovial fluid and at the articular surface, are likely key functional determinants of PRG4's boundary lubricating ability. PRG4 synthesis and secretion by chondrocytes could significantly contribute to the concentration of PRG4 within synovial fluid, in both homeostatic and pathological conditions where physiological regulators are present [14]. Although the amount of PRG4 bound to the surface does not appear to correlate with secretion rates, previous studies suggest surface bound PRG4 can be modulated by exchange with endogenous PRG4 in synovial fluid [19], and mechanical perturbation [18, 28]. Clarification of the spatial and temporal aspects of PRG4 metabolism within the joint, particularly at the articular surface, would further the understanding of PRG4's contribution to the low-friction properties of articular cartilage, and possibly lead to treatments to prevent loss of this function. Additionally, the combination of chemical and mechanical factors to stimulate PRG4 expression in chondrocytes near the articular surface may be useful for creating tissue engineered cartilage from isolated sub-populations [12] with a functional lubricating surface.

4.6 Acknowledgments

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CHAPTER 5

EFFECT OF SYNOVIAL FLUID ON BOUNDARY LUBRICATION OF ARTICULAR CARTILAGE

5.1 Abstract

Objective. The lubrication of articulating cartilage surfaces in joints occurs through several distinct modes. In the boundary mode of lubrication, load is supported by surface-to-surface contact, a feature that makes this mode particularly important for maintenance of the normally pristine articular surface. A boundary mode of lubrication is indicated by a kinetic friction coefficient being invariant with factors that influence formation of a fluid film, including sliding velocity and axial load. The objectives of this study were to 1) implement and extend an *in vitro* articular cartilage-on-cartilage lubrication test to elucidate the dependence of the friction properties on sliding velocity, axial load, and time, and establish conditions where a boundary mode of lubrication is dominant, and 2) determine the effects of synovial fluid on boundary lubrication using this test.

Methods. Fresh bovine osteochondral samples were analyzed in an annulus-on-disk rotational configuration, maintaining apposed articular surfaces in contact, to

determine static (μ_{static} and $\mu_{static, Neq}$) and kinetic ($<\mu_{kinetic}>$ and $<\mu_{kinetic, Neq}>$) friction coefficients, each normalized to the instantaneous and equilibrium (N_{eq}) normal loads, respectively.

Results. With increasing pre-sliding durations, μ_{static} and $\mu_{static, Neq}$ were similar, and increased up to 0.43±0.03 in PBS and 0.19±0.01 in SF, whereas $<\mu_{kinetic}>$ and $<\mu_{kinetic, Neq}>$ were steady. Over a range of sliding velocities of 0.1-1 mm/s and compression levels of 18% and 24%, $<\mu_{kinetic}>$ was 0.072±0.010 in PBS and 0.014±0.003 in SF, and $<\mu_{kinetic, Neq}>$ was 0.093±0.005 in PBS and 0.018±0.002 in SF.

Conclusion. A boundary mode of lubrication was achieved in a cartilage-on-cartilage test configuration. Synovial fluid functioned as an effective friction-lowering boundary lubricant for native articular cartilage surfaces.

5.2 Introduction

Articular cartilage normally serves as the low friction, wear resistant, load bearing tissue at the end of long bones in skeletal joints [42]. The articulation of cartilage against cartilage presents a major biomechanical challenge, with an individual typically taking 1-4 million steps each year [41]. Unfortunately, the pristine structure of the articular cartilage surface often deteriorates with aging and arthritis, becoming increasingly roughened and eroded, with development of pain and dysfunction, and progressing to osteoarthritis [6]. Thus, the extent and modes of the normal lubrication of articulating cartilage surfaces are important to understand.

A number of physicochemical modes of lubrication occur in synovial joints and have been classified as fluid film or boundary [2, 47]. The operative lubrication modes depend on the normal and tangential forces on the articulating tissues, on the relative rate of tangential motion between these surfaces, and on the time history of both loading and motion [13, 14]. The friction coefficient, μ, provides a quantitative measure, and is defined as the ratio of tangential friction force to the normal force. One type of fluid-mediated lubrication mode is hydrostatic. At the onset of loading and typically for a prolonged duration, the interstitial fluid within cartilage becomes pressurized, due to the biphasic nature of the tissue; fluid may also be forced into the asperities between articular surfaces through a weeping mechanism [30]. Pressurized interstitial fluid and trapped lubricant pools may therefore contribute significantly to the bearing of normal load with little resistance to shear force, facilitating a very low μ [2]. Also, at the onset of loading and/or motion, squeeze film, hydrodynamic, and

elastohydrodynamic types of fluid film lubrication occur, with pressurization, motion, and deformation acting to drive viscous lubricant from and/or through the gap between two surfaces in relative motion.

In contrast, in boundary lubrication, load is supported by surface-to-surface contact, and the associated frictional properties are determined by lubricant surface molecules. This mode has been proposed to be important because the apposing cartilage layers make contact over ~10% of the total area, and this may be where most of the friction occurs [33]. Furthermore, with increasing loading time and dissipation of hydrostatic pressure, lubricant-coated surfaces bear an increasingly higher portion of the load relative to pressurized fluid, and consequently, μ can become increasingly dominated by this mode of lubrication [30, 32]. A boundary mode of lubrication is indicated by values of μ during steady sliding being invariant with factors that influence formation of a fluid film, such as relative sliding velocity and axial load [11]. Boundary lubrication, in essence, mitigates stick-slip [32], and is therefore manifest as decreased resistance both to steady motion and the start-up of motion. The latter situation is relevant to load bearing articulating surfaces after prolonged compressive loading (e.g., sitting or standing *in vivo*) [36]. Typical wear patterns of cartilage surfaces [31] also suggest that boundary lubrication of articular cartilage is critical to the protection and maintenance of the articular surface structure.

A variety of time-dependent *in vitro* mechanical tests have been developed to assess the effectiveness and modes of articular cartilage lubrication. Since joints are subject to sequential periods of rest and motion, the transition to motion represents one lubrication challenge, and steady-state motion represents an additional lubrication

challenge. Analogously, friction coefficients can be measured at startup from a static condition, i.e., μ_{static}, or under steady sliding or kinetic conditions, $\mu_{kinetic}$, although most tests have focused on the latter. μ_{static} increases (e.g., from ~0.02 – 0.25) with increasing loading times (5 s – 45 min) for both cartilage-cartilage and cartilage-metal interfaces [13]. $\mu_{kinetic}$ is low (~ 0.001-0.05) at early times after loading where fluid pressurization is significant, for normal articulating cartilage surfaces [27-29]. Conversely, when fluid depressurization is allowed after compression of apposed articular surfaces [29], as well as between cartilage and glass [24, 37], $\mu_{kinetic}$ is higher (~0.1-0.6). $\mu_{kinetic}$ also depends on the rotational velocity with cartilage apposed to and rotated against steel [45].

Cartilage-on-cartilage lubrication tests provide a configuration mimicking certain aspects of naturally articulating surfaces. Lubrication tests of cartilage against artificial surfaces may reproduce some, but not all, of the molecular interactions that are operative in physiological articulation [4]. The $\mu_{kinetic}$ of cartilage against an artificial surface can vary significantly (e.g. 0.14 for polystyrene [37] vs. 0.28 for glass [24]), suggesting that the surface apposing the articular cartilage is an important determinant of friction. Cartilage-on-cartilage tests may be performed in a sliding or rotational configuration, resulting in areas of contact between surfaces under relative motion; such contact areas may be either moving or constant, respectively. While the sliding test configuration models certain aspects of physiological kinematics [26], the rotational configuration has advantages for examining putative boundary lubricants of articular cartilage at a like interface [29]. In the sliding configuration, with a moving contact area, both fluid film and boundary lubrication are generally operative due to

fluid pressurization and exudation, even at relatively slow sliding velocities [1, 3]. In the rotational configuration, ploughing friction losses are minimized because the apposed surfaces remain in contact [5], and fluid pressure effects are minimal at relatively slow velocities after the initial pressure dissipates. Furthermore, with the use of an annular geometry [10, 16, 29], the variation in sliding velocity is reduced (due to its proportionality to the radius), as is the time required for fluid depressurization. Using this annulus-on-disk configuration for a cartilage-cartilage interface with nasal septal cartilage, Davis showed that synovial fluid (SF) lubricated better than Gey's balanced salt solution [10]. With articular cartilage samples, Malcom and Fung also found that SF lubricated static and dynamically loaded samples, after step loading and partial fluid depressurization, better than phosphate buffered saline (PBS) [16, 29]. Thus, the annulus-on-disk rotational test configuration appears to be advantageous for studying boundary lubrication at an articular cartilage-on-cartilage interface, possibly modulated by SF.

The objectives of this study were to 1) implement and extend an *in vitro* articular cartilage-on-cartilage annulus-on-disk lubrication test to elucidate the dependence of the friction properties on sliding velocity, axial load, and time, and establish conditions where a boundary mode of lubrication is dominant, and 2) determine the effects of synovial fluid on boundary lubrication using this test.

5.3 Materials and Methods

Materials. Skeletally mature adult bovine stifle joints (1-2 years old) were obtained as described previously [46]. Bovine SF was aspirated from synovial joints within 10-15 min of slaughter, visually inspected to ensure no blood contamination, then aliquoted and stored at -80°C for several months before use.

Sample Preparation. Osteochondral samples were prepared from the patellofemoral groove from four joints (Fig. 5.1A), in a manner similar to that described previously [25]. Osteochondral blocks were isolated first, and then osteochondral samples (n = 16) were cut from the blocks using a low speed drill press with custom stainless steel coring bits, using PBS at 4°C for irrigation. Each sample consisted of an osteochondral core, radius = 6 mm, and an apposed osteochondral annulus (outer radius, R_o = 3.2 mm, and inner radius, R_i = 1.5 mm), both with central holes (radius = 0.5 mm) drilled down into and exiting the bone to facilitate fluid depressurization (Fig. 5.1B). (Pilot studies indicated that inclusion of these holes reduced the time to attain 50% stress relaxation by ~10%). The cartilage thickness of each core and annulus were then measured with digital calipers at four equally spaced locations around the circumferences and averaged, and the overall cartilage thickness was taken as the sum of the two average thicknesses (3.32±0.30 mm, mean±SD for all 16 samples). Samples were used without prior freezing to preserve lubrication properties [29], and bathed in test lubricant, completely immersing the cartilage (Fig. 5.1C), at 4°C for 24-48 hr prior to lubrication testing.

Lubrication Test Setup. For lubrication testing, the sample core and annulus were placed in apposition, compressed axially, and subjected to relative rotation. Samples were tested in an ELF 3200 (Bose EnduraTEC, Minnetonka, MN) with custom sample fixtures, axial and rotational actuators, an internal sensor for axial displacement (±6.250 mm range), and sensors for axial load (N) and torque (τ) (with ranges of ±45.0 N, and ±70.0 N•mm, respectively). Samples were brought to room temperature then placed concentrically in the ELF 3200, with the core on the rotational actuator below, and the annulus on the sensors and axial actuator above (Fig. 5.1D). A test lubricant reservoir was formed by circumferentially securing an inert silicon rubber tube around the core and adding ~0.5 ml of test lubricant, completely immersing both cartilage test surfaces. The samples were then brought into contact, defined as the axial position half-way between the points of initial and final contact, determined as the positions of maximum and minimum N, respectively, measured during one complete revolution. The sample surfaces were aligned normal to the rotation axis, as judged by the axial distance between the points of initial and final contact being < 0.1 mm (i.e., < 4% of the thickness of the apposed articular cartilage). During rotational testing, the change in radial distance between the outer edge of the core and annulus cartilage surfaces was estimated to be 0.0-0.5 mm. Even with the highest value, the contact area during rotation was calculated to change by only ~13%, indicating that the contacting areas were approximately constant.

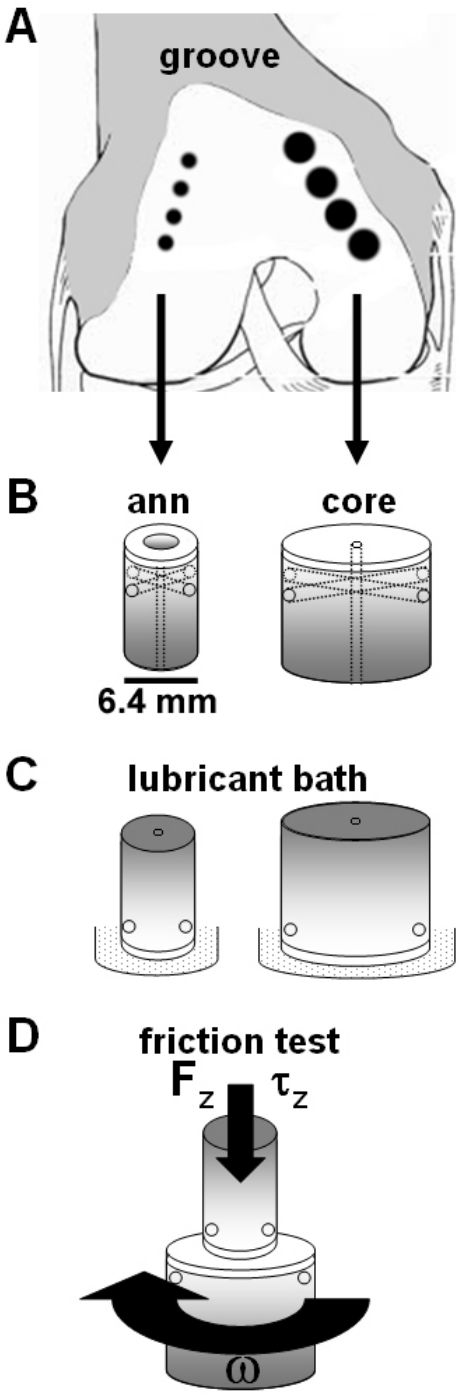

Figure 5.1: Diagram of harvest location, specimen preparation and lubricant bath incubation, and friction testing. Blocks were harvested from the patellofemoral groove of mature bovine stifle joints (**A**) from which osteochondral annulus (ann) and core sample (**B**) were prepared. Sample pairs were incubated in the test bath solution (PBS or SF) at 4°C for 24-48 hr (**C**) prior to friction testing (**D**).

Experimental Design. To determine the test conditions in which boundary lubrication was the dominant mode at the articular cartilage-on-cartilage interface, the dependence of frictional properties on post-compression pause durations, compression level, and sliding velocity was examined. Specifically, the effects of stress relaxation duration (T_{sr}, the duration allowed for fluid depressurization after the sample is compressed), compression ($1-\Lambda_Z$, where Λ_Z is the stretch ratio [16]), effective sliding velocity ($v_{eff} = \omega R_{eff}$, where ω is the angular frequency, in rad/s, and R_{eff} is the effective radius calculated to be $2/3 \bullet [(R_o^3 - R_i^3)/ R_o^2 - R_i^2)] = 2.4$ mm by integrating the shear stress distribution over the annular contact area [29]), and pre-sliding duration (T_{ps}, the duration the sample is stationary prior to rotation), on the lubrication properties of articular cartilage were assessed with PBS, and then SF, as lubricant solutions (Fig. 5.2). Samples were first bathed in a small volume (~1 ml) of PBS, and then tested for lubrication properties in PBS. Samples were subsequently bathed in SF, followed by lubrication testing in SF. Due to the potential structural, and therefore functional, alterations of lubricant molecules within SF, protease inhibitors were not included in the test lubricants. Control studies indicated no deterioration of friction properties over the duration of the test period, as friction coefficients (described below) of samples, stored at 4°C, tested in SF (at $1-\Lambda_Z = 18\%$ and 24%, with $T_{sr} = 60$ min, $v_{eff} = 0.3$ mm/s, and $T_{ps} = 120$ s) on day 4 were similar to those measured on day 1 (97±8% for μ_{static} and 88±9% for $<\mu_{kinetic}>$, n=4). Preliminary tests also confirmed that testing in PBS then SF did not affect measured values in SF. Mechanical properties appeared to be maintained as well since equilibrium N values (N_{eq}) attained

in the 2nd test lubricant were within ~6% of the first. Data was collected at 20 Hz during the v_{eff} = 3 mm/s test revolutions, and 10 Hz for all others.

Effect of Stress Relaxation. Samples (n = 4) were compressed at a constant rate of 0.002 mm/s to $1-\Lambda_Z$ = 18% of the total cartilage thickness (Fig. 5.2A), then tested by rotating +2 revolutions, immediately followed by -2 reset revolutions at v_{eff} = 0.3 mm/s (which is on the order of that used in other test configurations [20] and has been found to maintain a boundary or mixed mode of lubrication [45]). Test revolutions were then performed at T_{sr} = 2, 7, 13, 29, 44, and 60 min, with T_{ps} = 120 s between each (Fig. 5.2B). The 60 min duration was based on a characteristic time constant $t_{char} = l^2/(H_A k_p)$, where l is the characteristic length, $(R_o - R_i)/2$ mm = 0.85 mm, H_A is the modulus, 0.31 MPa, and k_p is the hydraulic permeability, 10^{-15} m^2/Pa•s [15, 34, 46], yielding t_{char} = 45 min, as validated experimentally, below. Pilot studies indicated T_{ps} = 120 s was sufficient to measure differences between μ_{static} and $<\mu_{kinetic}>$, defined below, such that the stick-slip process mitigated by boundary lubrication could be observed. Samples were then unloaded and held at $1-\Lambda_Z$ = 0% for 120 min to allow for creep. Samples were then compressed to $1-\Lambda_Z$ = 18% again, and the test sequence was then repeated in the opposite direction of rotation.

Effect of Sliding Velocity & Compression. Samples (n = 4) were compressed to $1-\Lambda_Z$ = 12% of the total cartilage thickness (Fig. 5.2C), as described above, and allowed T_{sr} = 60 min for stress relaxation and fluid depressurization. The test sequence was initiated by conditioning the sample by rotating +2 revolutions and reset with -2 revolutions at v_{eff} = 3 mm/s. Samples were then tested by rotating +2 revolutions, immediately followed by -2 reset revolutions at v_{eff} of 3, 1, 0.3, 0.1, and

then 3 mm/s, with T_{ps} = 120 s between each v_{eff} (Fig. 5.2D). The test sequence was then repeated in the opposite direction of rotation. Samples were subsequently compressed at the same rate (0.002 mm/s) to 1-Λ_Z = 18% and then 24% of the total cartilage thickness and the entire test sequence was repeated at each compression level in both directions of rotation.

Effect of Pre-Sliding Duration. Samples (n = 4) were compressed to 1-Λ_Z = 18% of the total cartilage thickness at 0.002 mm/s (Fig. 5.2E) and allowed to stress relax as described above. The test sequence was initiated in a similar manner as well, except with v_{eff} = 0.3 mm/s. The samples were then tested by rotating +2 revolutions, immediately followed by -2 reset revolutions with v_{eff} = 0.3 mm/s and T_{ps} = 3600, 1200, 120, 12, and 1.2 s (Fig. 5.2F). The test sequence was then repeated with rotation in the opposite direction.

Role of Fluid Depressurization During Rotation. With SF as the test lubricant, samples (n = 4) were compressed to 1-Λ_Z = 18% of the total cartilage thickness (Fig. 5.2G), and allowed to stress relax as described above. The test sequence was initiated as described above, with v_{eff} = 3 mm/s. After T_{ps} = 120 s, samples were then subjected to +2.5 revolutions with v_{eff} = 3 mm/s, and finally monitored for another 60 min, to assess possible stress relaxation, indicative of fluid depressurization (Fig. 5.2H).

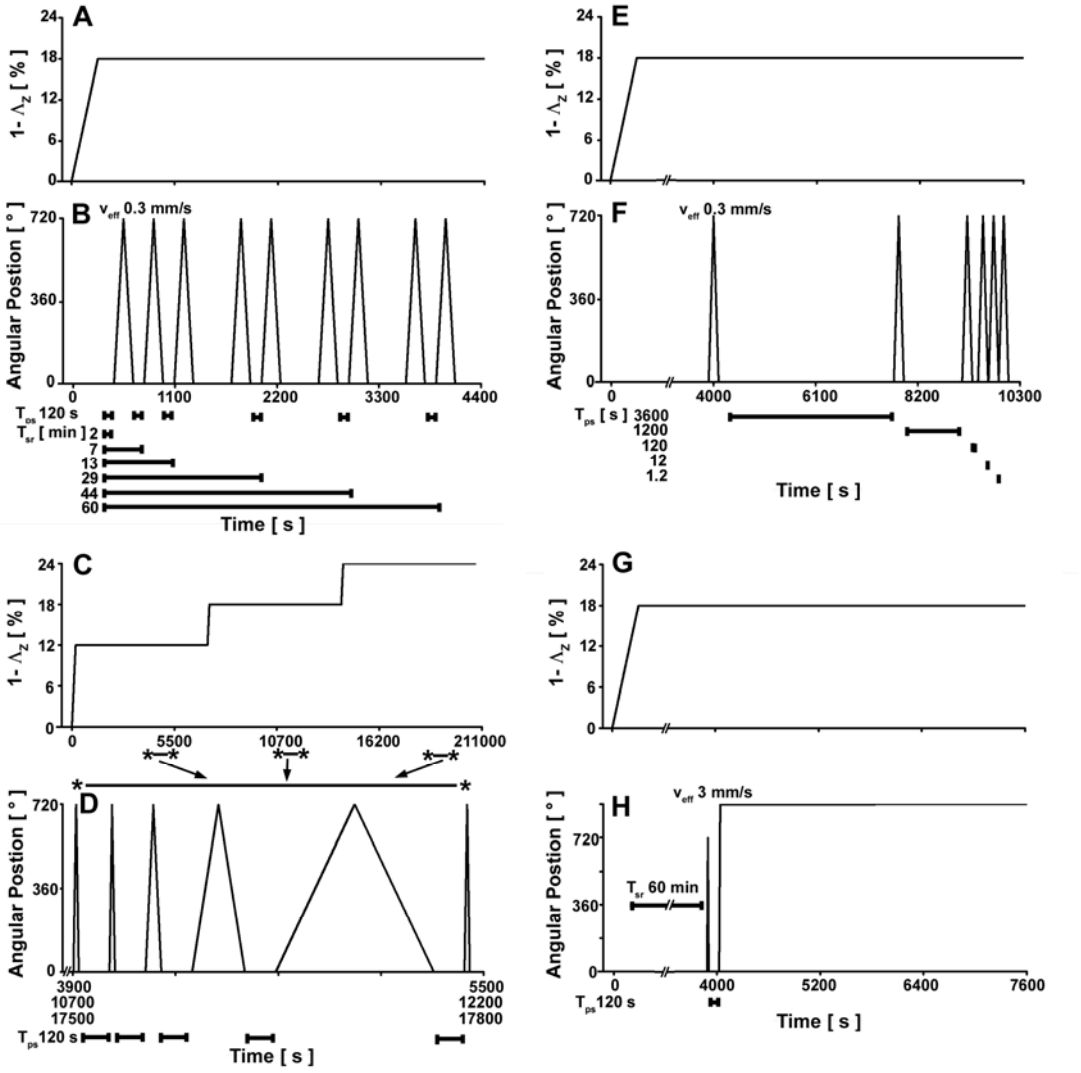

Figure 5.2: Lubrication test protocols defined by stress relaxation duration (T_{sr}), compression ($1\text{-}\Lambda_Z$), effective sliding velocity (v_{eff}), and pre-sliding duration (T_{ps}). Samples were compressed axially by $1\text{-}\Lambda_Z$ = 18% (**A, E, G**), or 12%, 18%, and 24% (**C**) of the total cartilage thickness. Rotational test protocols were then respectively used to determine the effects of T_{sr} (**B**), $1\text{-}\Lambda_Z$ and v_{eff} (**D**), T_{ps} (**F**), and fluid depressurization (**H**) on the lubrication properties of articular cartilage for PBS and/or SF lubricants. Schematics (**B, D, F**), only shows + test revolution sequence, as the identical - test sequence proceeded subsequently with revolutions in the opposite direction.

Data Analysis. To evaluate the lubrication properties of the articular cartilage in test lubricants (PBS and SF), four friction coefficients (μ) of the form $\mu = \tau / (R_{eff} \bullet N)$ were calculated, where τ is torque, N is axial load, and R_{eff} is effective radius, described above. Classical static (μ_{static}) and kinetic ($<\mu_{kinetic}>$) friction coefficients were calculated from the instantaneous μ described above. μ_{static} was calculated as the peak value of μ, just after (within 10°) the start of rotation, and $<\mu_{kinetic}>$ was calculated from μ averaged during the second complete revolution of the test sample. Another static friction coefficient, $\mu_{static, Neq}$, was calculated using the peak $| \tau |$, also measured within 10° of the start of rotation, and the axial load at the end of the 60 min stress relaxation period, N_{eq}. In all of the above tests except the first (which examined the effect of stress relaxation), another kinetic friction coefficient, $<\mu_{kinetic, Neq}>$, was calculated using the $| \tau |$ averaged during the second complete revolution of the test sample, and N_{eq}. The values of μ_{static}, $\mu_{static, Neq}$, $<\mu_{kinetic}>$, and $<\mu_{kinetic, Neq}>$ were then averaged for the + and – revolutions in each test to account for potential directional effects on τ measurements. The normal stress (σ), in units of MPa, was calculated as $| N | / (\pi [R_o^2 - R_i^2])$. The equilibrium stress values (σ_{eq}) were calculated after $T_{sr} = 60$ min; the peak stress (σ_{peak}) values were calculated from the peak $| N |$ during rotation, and averaged for the + and – revolutions.

Data are presented as the mean ± SEM. Repeated measures analysis of variance (ANOVA) was used to assess the effect of test lubricant, T_{sr}, $1-\Lambda_Z$, v_{eff}, and T_{ps} on μ_{static}, $\mu_{static, Neq}$, $<\mu_{kinetic}>$, and $<\mu_{kinetic, Neq}>$. Where there were three factors, and test lubricant had a significant effect, data for each lubricant were analyzed further

using a 2-factor ANOVA. Statistical analysis was implemented with Systat 10.2 (Systat Software, Inc., Richmond, CA).

Table 5.1: Symbols used for variables and parameters.

variable or parameter	symbol
stretch ratio	Λ_z
kinetic friction coefficient	$\mu_{kinetic}$
kinetic friction coefficient normalized by equilibrium axial load	$\mu_{kinetic,\ Neq}$
static friction coefficient	μ_{static}
static friction coefficient normalized by equilibrium axial load	$\mu_{static,\ Neq}$
axial load	N
equilibrium axial load	N_{eq}
effective radius	R_{eff}
inner radius	R_i
outer radius	R_o
normal stress	σ
equilibrium normal stress	σ_{eq}
peak normal stress	σ_{peak}
axial torque	τ
time	t
pre-sliding duration	T_{ps}
stress relaxation duration	T_{sr}
effective sliding velocity	v_{eff}

5.4 Results

Lubrication Test Characterization. The sample preparation and lubrication test setup enabled precise measurements of τ and N during the various tests. Typical $|\tau|$ values ranged from 0.1 to 5 N•mm, with transient torque values immediately after the start of the test revolution (corresponding to μ_{static} and $\mu_{static,\ Neq}$) being clearly distinguishable (with the torque sensor precision of ±0.01 N•mm) from the steady state values by the beginning of the second test revolution. Typical | N | values ranged from 1-10 N during the test, which were clearly resolved by the axial load sensor (precision of ±0.1 N) with feedback control of the axial displacement (precision of ±0.001 mm). Only the raw data from the +2 revolutions of the tests are shown subsequently, for brevity, since reduction of data from the -2 revolutions of the tests gave friction coefficients that were similar on average (within 1±14%, mean±SD) to those determined from the +2 revolutions.

Effect of Stress Relaxation. The $|\tau|$ (Fig. 5.3AB) and | N | (Fig. 5.3CD) during the 2 test revolutions varied with T_{sr}. In both the PBS and SF test lubricants, $|\tau|$ and | N | decreased qualitatively as T_{sr} increased from 2 to 7 min. $|\tau|$ was greater in PBS than SF, while | N | was similar in PBS and SF. The peak $|\tau|$ (see insets of Fig. 5.3AB) dissipated to relatively steady values by 360°. The | N | was cyclical during the 2 test revolutions, peaking at approximately 180° and 540°, with the amplitude being greater at 180° compared to 540°. The σ_{peak} values attained at $1-\Lambda_Z = 18\%$ in PBS and SF ranged (for T_{sr} = 60 to 2 min) from 0.42±0.05 to 0.21±0.03 MPa and 0.46±0.06 to

0.23±0.03 MPa, respectively. The σ_{eq} values attained at T_{sr} = 60 min and 1-Λ_Z = 18% in PBS and SF were both 0.10±0.01 MPa.

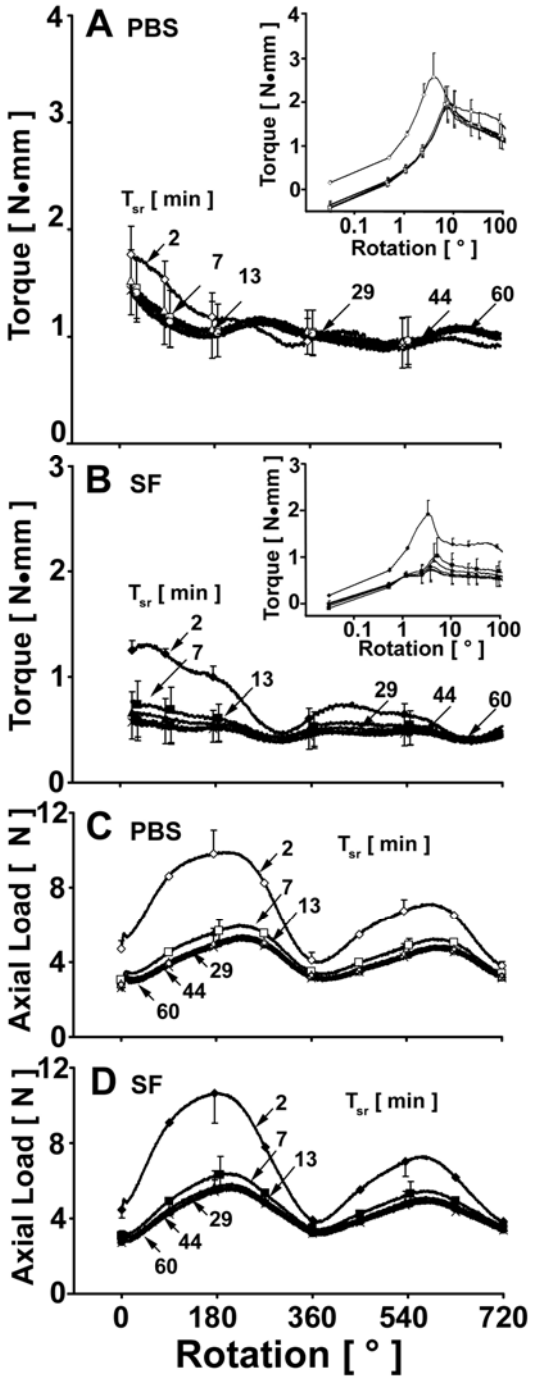

Figure 5.3: Torque (**A, B** with log scale insets to show values at small rotation angles) and axial load (**C, D**) measurements versus rotation in test baths of PBS and SF at 18% compression ($1-\Lambda_Z$) after 2, 7, 13, 29, 44, 60 min stress relaxation duration (T_{sr}), at an effective sliding velocity (v_{eff}) of 0.3 mm/s with a 120 s pre-sliding duration (T_{ps}). Mean ± SEM, n=4.

Friction was modulated by test lubricant and T_{sr} (Fig. 5.4). μ_{static} varied with test lubricant (being higher in PBS than SF, $p<0.05$) and T_{sr} ($p<0.001$), with an interaction effect ($p<0.001$) (Fig. 5.4A). In PBS, μ_{static} increased with T_{sr} and ranged from 0.18 to 0.25. In SF, μ_{static} averaged 0.11. Similarly, $\mu_{static,\ Neq}$ varied with test lubricant (also being higher in PBS than SF, $p<0.05$) and T_{sr} ($p<0.001$), without an interaction effect ($p=0.06$) (Fig. 5.4B). However, contrary to μ_{static}, $\mu_{static,\ Neq}$ decreased with increasing T_{sr}, and ranged from 0.37 to 0.28 in PBS, and 0.21 to 0.11 in SF. Nevertheless, in both PBS and SF, μ_{static} and $\mu_{static,\ Neq}$ converged to a similar value as $T_{sr} \rightarrow 60$ min. Thus, in the subsequent tests where $T_{sr} = 60$ min, only $\mu_{static,\ Neq}$ values are reported since μ_{static} values were similar (on average within 5±19%, mean±SD), due to $|N_{eq}|$ being similar to $|N|$ immediately after the start of rotation. Lastly, $<\mu_{kinetic}>$ varied with T_{sr} ($p<0.001$), with an interaction effect ($p<0.001$) and without an effect of test lubricant ($p=0.07$) (Fig. 5.4C). $<\mu_{kinetic}>$ increased slightly with T_{sr} and was greater in PBS than SF, ranging from 0.065 to 0.096 in PBS, and 0.029 to 0.035 in SF.

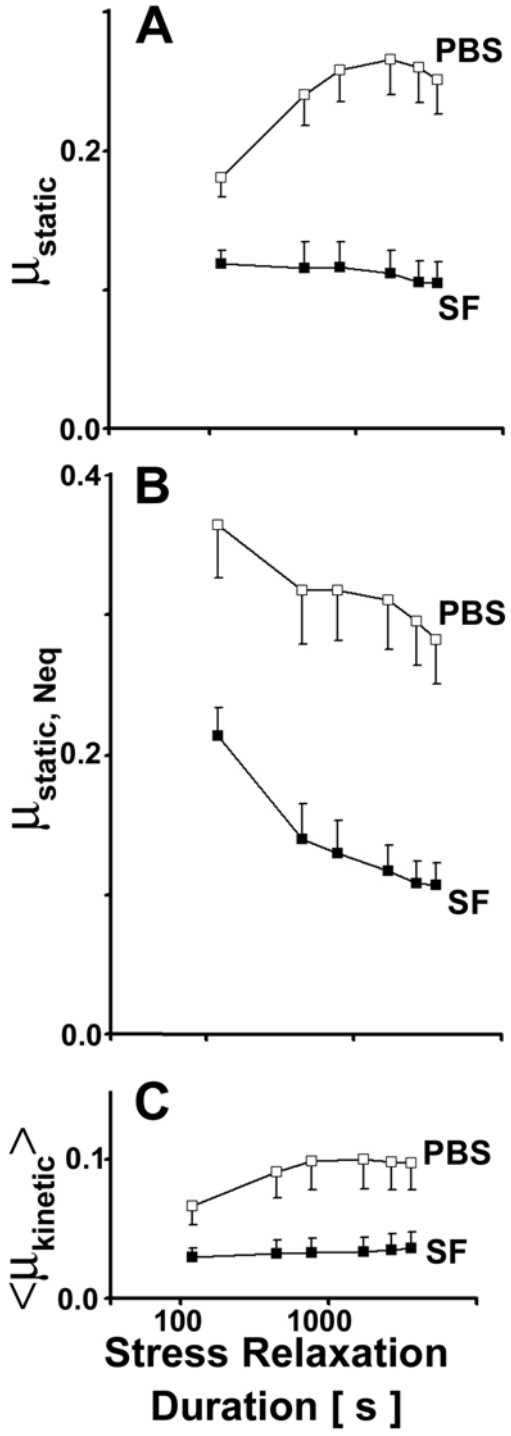

Figure 5.4: Static, μ_{static} (**A**), $\mu_{static,\ Neq}$ (**B**), and kinetic, $<\mu_{kinetic}>$ (**C**) friction coefficients in PBS and SF at 18% compression ($1-\Lambda_Z$) after 2, 7, 13, 29, 44, 60 min stress relaxation duration (T_{sr}), at an effective sliding velocity (v_{eff}) of 0.3 mm/s with a 120 s pre-sliding duration (T_{ps}). Mean ± SEM, n=4.

Effect of Sliding Velocity & Compression. The $|\tau|$ (Fig. 5.5AB) and $|N|$ (Fig. 5.5CD) during the 2 test revolutions at $1\text{-}\Lambda_Z = 18\%$ varied with v_{eff}. In both the PBS and SF test lubricants, $|\tau|$ and $|N|$ increased qualitatively with v_{eff}. $|\tau|$ was greater in PBS then SF, while $|N|$ was similar in PBS and SF. The peak $|\tau|$ (see insets of Fig. 5.5AB) dissipated to an approximately steady state value by 360°, as indicated by the ratio of $\tau_{360\text{-}720°}$ to $\tau_{360°}$ being 95±10% (mean±SD). The $|N|$ was cyclical during the 2 test revolutions, peaking at approximately 180° and 540°, as noted above (Fig. 5.3CD). The respective σ_{peak} values attained in PBS and SF ranged from 0.23±0.02 to 0.11±0.01 MPa and 0.31±0.03 to 0.14±0.01 MPa (for v_{eff} = 3 to 0.1 mm/s) at $1\text{-}\Lambda_Z =$ 12%, 0.24±0.02 to 0.13±0.01 MPa and 0.33±0.04 to 0.16±0.02 MPa at $1\text{-}\Lambda_Z = 18\%$, and 0.26±0.02 to 0.14±0.02 MPa and 0.36±0.05 to 0.18±0.02 MPa at $1\text{-}\Lambda_Z = 24\%$. The respective σ_{eq} values attained in PBS and SF were 0.07±0.01 MPa and 0.07±0.01 MPa at $1\text{-}\Lambda_Z = 12\%$, 0.09±0.01 MPa and 0.11±0.01 MPa at $1\text{-}\Lambda_Z = 18\%$, and 0.11±0.02 MPa and 0.14±0.02 MPa at $1\text{-}\Lambda_Z = 24\%$. Thus, after initial fluid depressurization, $|\tau|$ transients dissipated by the second test revolution, and $|N|$ measurements were generally unaffected by the test lubricant at the various $1\text{-}\Lambda_Z$ and v_{eff}.

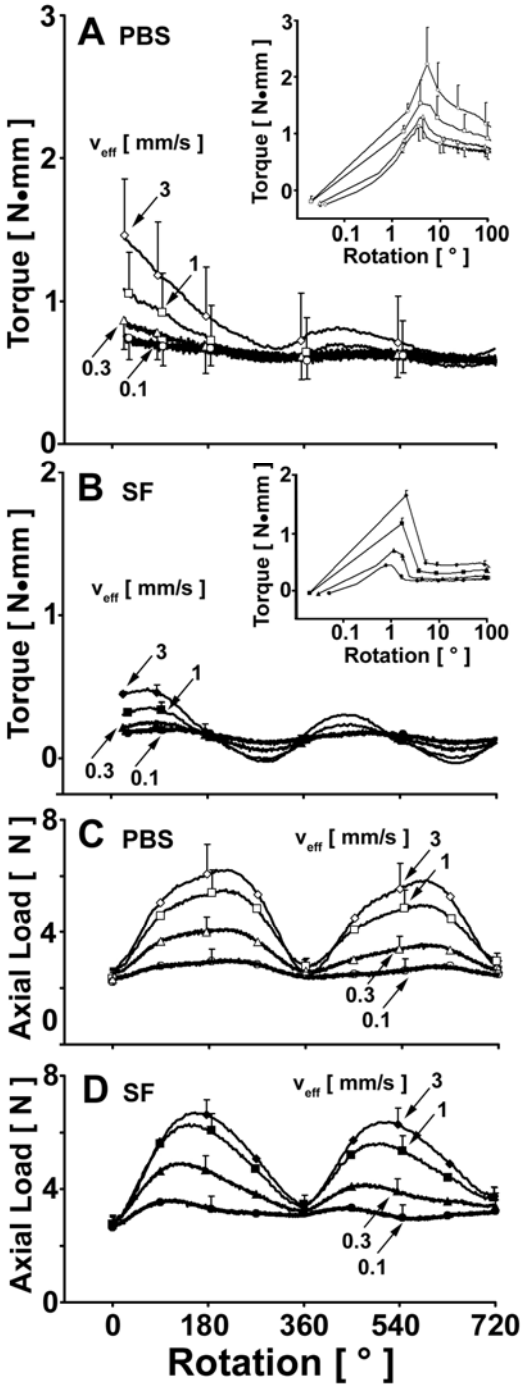

Figure 5.5: Torque (**A, B** with log scale insets to show values at small rotation angles) and axial load (**C, D**) measurements versus rotation in test baths of PBS and SF at 18% compression ($1-\Lambda_Z$) after 60 min stress relaxation duration (T_{sr}), at effective sliding velocities (v_{eff}) of 3, 1, 0.3, and 0.1 mm/s with a 120 s pre-sliding duration (T_{ps}). Mean ± SEM, n=4.

Friction was modulated by test lubricant, $1-\Lambda_Z$, and v_{eff} (Fig. 5.6). $\mu_{static,\ Neq}$ varied with test lubricant ($p<0.05$), $1-\Lambda_Z$ ($p<0.05$), and v_{eff} ($p<0.001$), with an interaction effect between $1-\Lambda_Z$ and v_{eff} ($p<0.001$) (Fig. 5.6A). For tests in PBS, $\mu_{static,\ Neq}$ varied with v_{eff} ($p<0.001$) and an interaction between $1-\Lambda_Z$ and v_{eff} ($p<0.05$). For tests in SF, $\mu_{static,\ Neq}$ varied with $1-\Lambda_Z$, v_{eff} (both $p<0.001$), and an interaction effect ($p<0.05$). Values of $\mu_{static,\ Neq}$ were greater when samples were tested in PBS than when samples were tested in SF, and increased with v_{eff}, ranging from 0.21 to 0.41 in PBS, and 0.074 to 0.28 in SF, at $1-\Lambda_Z = 18\%$. Conversely, $\mu_{static,\ Neq}$ decreased with $1-\Lambda_Z$ at every v_{eff}, ranging from 0.23 to 0.28 and 0.10 to 0.16 in PBS and SF, respectively, at $v_{eff} = 0.3$ mm/s. This variation with increasing $1-\Lambda_Z$ was attributable to an increase in peak $|\ \tau\ |$ that was relatively small compared to the increase in $|\ N_{eq}\ |$.

$<\mu_{kinetic}>$ varied markedly with test lubricant (being higher in PBS than SF, $p<0.05$) and slightly with $1-\Lambda_Z$ ($p<0.05$) but not v_{eff} ($p=0.16$), with an interaction effect between test lubricant and $1-\Lambda_Z$ ($p<0.01$) (Fig. 5.6B). In PBS, $<\mu_{kinetic}>$ varied with $1-\Lambda_Z$ ($p<0.05$), increasing from 0.043 to 0.079 at 3 mm/s, and tended to decrease with v_{eff}. In SF, $<\mu_{kinetic}>$ varied with v_{eff} ($p<0.01$), remaining steady at 0.014 at all $1-\Lambda_Z$ and the lower $v_{eff} = 0.1 - 1$ mm/s. $<\mu_{kinetic,\ Neq}>$ varied with test lubricant ($p<0.05$) but not significantly with $1-\Lambda_Z$ ($p=0.28$) or v_{eff} ($p=0.56$), with interaction effects between test lubricant and $1-\Lambda_Z$ ($p<0.001$), $1-\Lambda_Z$ and v_{eff} ($p<0.01$), and test lubricant, $1-\Lambda_Z$ and v_{eff} ($p<0.001$) (Fig. 5.6C). Specifically, in PBS, $<\mu_{kinetic,\ Neq}>$ varied with $1-\Lambda_Z$ ($p<0.01$) and an interaction between $1-\Lambda_Z$ and v_{eff} ($p<0.001$); in SF, $<\mu_{kinetic,\ Neq}>$

varied with $1-\Lambda_Z$ ($p<0.05$) and v_{eff} ($p<0.01$). In PBS, $<\mu_{kinetic,\ Neq}>$ was greater than in SF, increased with $1-\Lambda_Z$ only at the larger v_{eff}, ranging from 0.080 to 0.13 at 3 mm/s, and remained steady at 0.090 at all $1-\Lambda_Z$ and the lower v_{eff} = 0.1 – 1 mm/s. Similarly in SF, $<\mu_{kinetic,\ Neq}>$ remained steady, at all $1-\Lambda_Z$ and the lower v_{eff} = 0.1 – 1 mm/s, at 0.020. Friction coefficients calculated at the first v_{eff} = 3 mm/s were reproduced with the second v_{eff} = 3 mm/s, reaching values of 100±8%, 92±7%, 100±10%, and 111±13% (mean±SD) for μ_{static}, $\mu_{static,\ Neq}$, $<\mu_{kinetic}>$, and $<\mu_{kinetic,\ Neq}>$ respectively. Therefore, μ_{static}, $\mu_{static,\ Neq}$, $<\mu_{kinetic}>$, and $<\mu_{kinetic,\ Neq}>$ were generally unaffected by the sequence of v_{eff} tested.

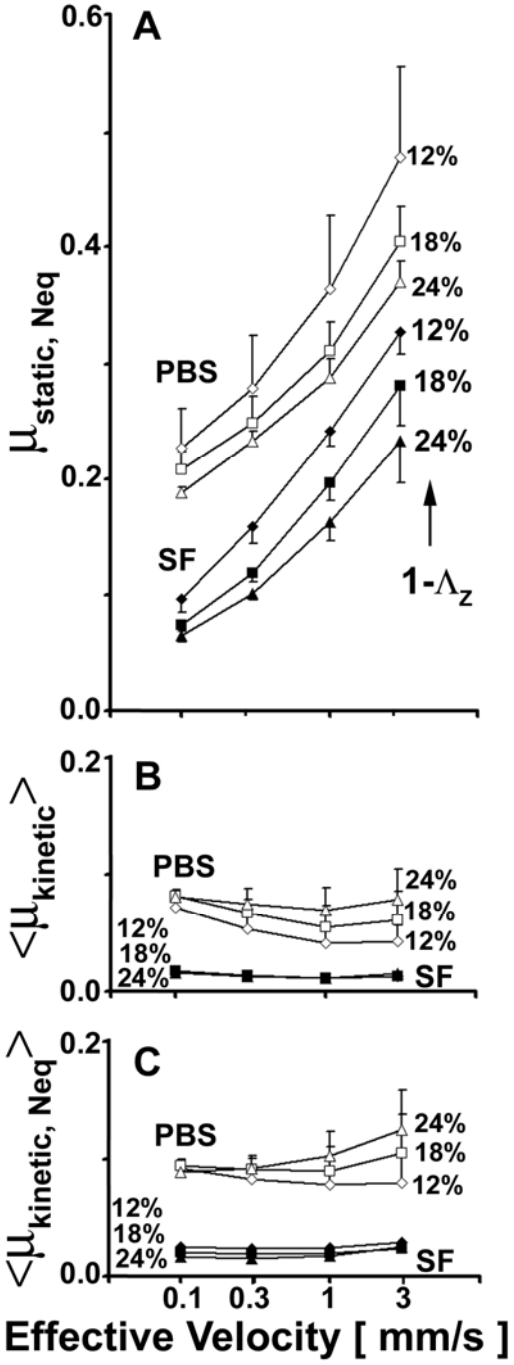

Figure 5.6: Static, $\mu_{static,\ Neq}$ (**A**), and kinetic, $<\mu_{kinetic}>$ (**B**), $<\mu_{kinetic,\ Neq}>$ (**C**) friction coefficients in PBS and SF at 12%, 18%, and 24% compression ($1-\Lambda_Z$) after 60 min stress relaxation duration (T_{sr}), at effective sliding velocities (v_{eff}) of 3, 1, 0.3, and 0.1 mm/s with a 120 s pre-sliding duration (T_{ps}). Mean ± SEM, n=4.

Effect of Pre-Sliding Duration. The $|\tau|$ (Fig. 5.7AB) and $|N|$ (Fig. 5.7CD) during the 2 test revolutions varied with T_{ps}. In both the PBS and SF test lubricants, $|\tau|$ and $|N|$ increased qualitatively with T_{ps}. $|\tau|$ was greater in PBS than SF, while $|N|$ was similar in PBS and SF. Consistent with the data above (Figs. 5.3AB and 5.5AB), the peak $|\tau|$ (see insets of Fig. 5.7AB), dissipated to an approximately steady-state value by 360°. Also consistent with the data above (Figs. 5.3CD and 5.5CD), the $|N|$ was cyclical during the 2 test revolutions, peaking at approximately 180° and 540°. The respective σ_{peak} values attained at $1-\Lambda_Z = 18\%$ in PBS and SF ranged (for T_{ps} = 3600 to 1.2 s) from 0.36±0.04 to 0.22±0.04 MPa and 0.40±0.02 to 0.24±0.03 MPa. The σ_{eq} values attained at $1-\Lambda_Z = 18\%$ in PBS and SF were both 0.13±0.01 MPa.

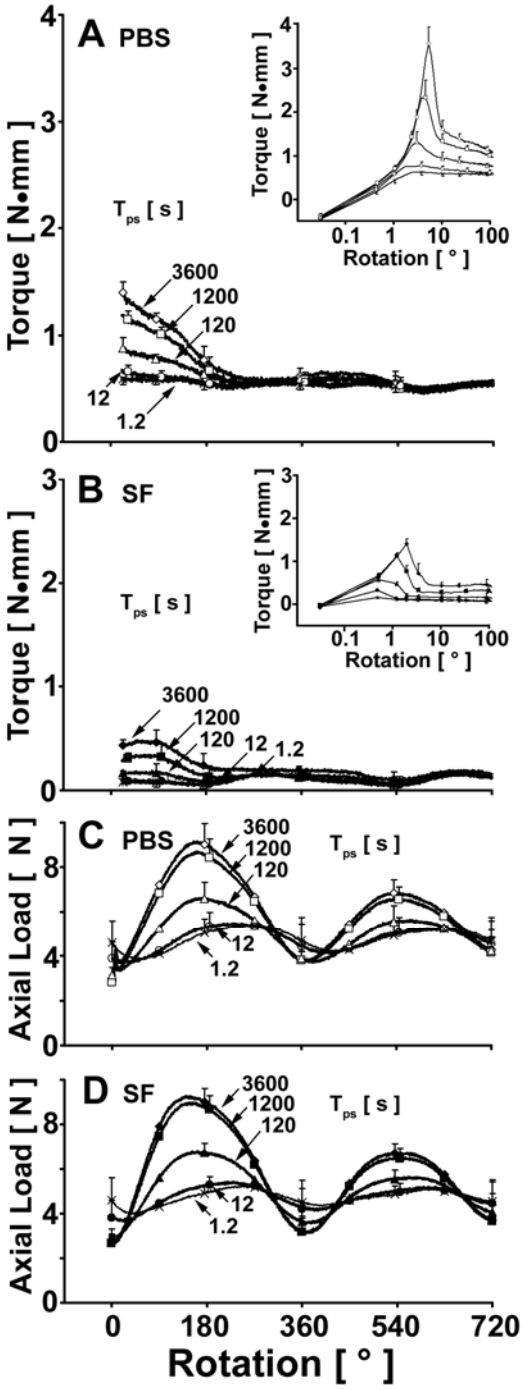

Figure 5.7: Torque (**A, B** with log scale insets to show values at small rotation angles) and axial load (**C, D**) measurements versus rotation in test baths of PBS and SF at 18% compression ($1-\Lambda_Z$) after 60 min stress relaxation duration (T_{sr}), at an effective sliding velocity (v_{eff}) of 0.3 mm/s with a 3600, 1200, 120, 12, and 1.2 s pre-sliding duration (T_{ps}). Mean ± SEM, n=4.

Friction was modulated by test lubricant and T_{ps} (Fig. 5.8). $\mu_{static,\ Neq}$ varied with test lubricant ($p<0.01$) and T_{ps} ($p<0.001$), with an interaction effect ($p<0.001$) (Fig. 5.8A). Values of $\mu_{static,\ Neq}$ were greater in PBS than SF, and increased with T_{ps}, ranging from 0.091 to 0.43 in PBS, and 0.021 to 0.19 in SF. $<\mu_{kinetic}>$ varied with test lubricant ($p<0.01$) but not T_{ps} ($p=0.87$), with no interaction effect ($p=0.37$) (Fig. 5.8B). $<\mu_{kinetic}>$ in PBS, 0.054, was greater than that in SF, 0.012. $<\mu_{kinetic,\ Neq}>$ varied with test lubricant ($p<0.001$) and T_{ps} ($p<0.05$) with no interaction effect ($p=0.91$) (Fig. 5.8C). Similar to $<\mu_{kinetic}>$, values of $<\mu_{kinetic,\ Neq}>$ were greater in PBS than SF, and increased slightly with T_{ps}, ranging from 0.077 to 0.082 (mean = 0.079) in PBS, and 0.017 to 0.023 (mean = 0.019) in SF. In both PBS and SF, $\mu_{static,\ Neq}$ appeared to approach $<\mu_{kinetic}>$, and $<\mu_{kinetic,\ Neq}>$, asymptotically as $T_{ps} \rightarrow 0$.

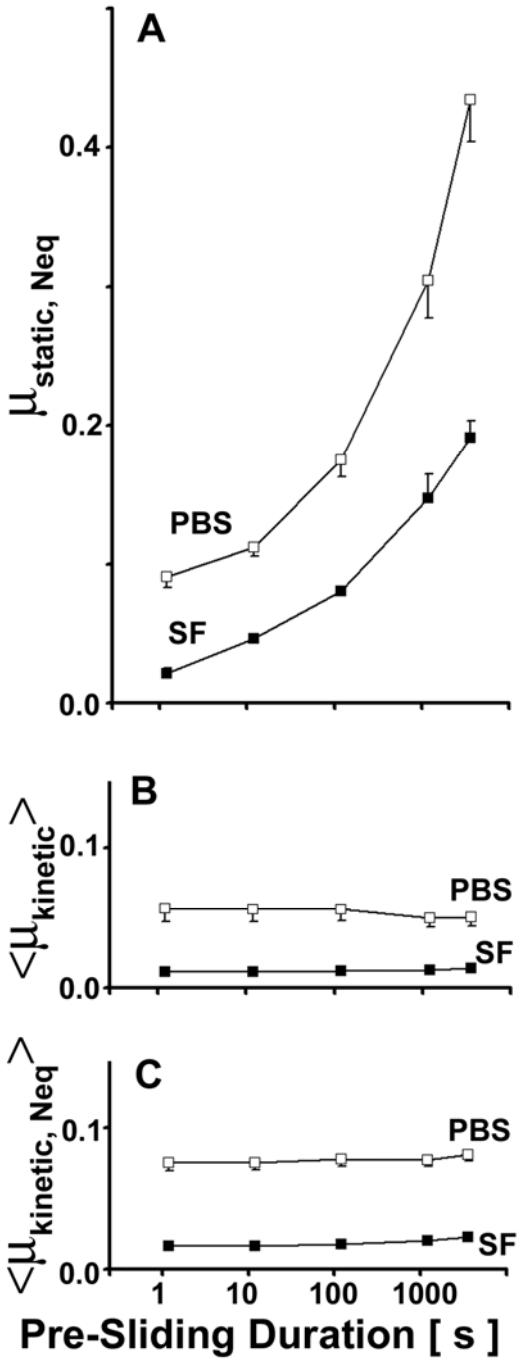

Figure 5.8: Static, $\mu_{static,\ Neq}$ (**A**), and kinetic, $<\mu_{kinetic}>$ (**B**), $<\mu_{kinetic,\ Neq}>$ (**C**) friction coefficients in PBS and SF at 18% compression ($1-\Lambda_Z$) after 60 min stress relaxation duration (T_{sr}), at an effective sliding velocity (v_{eff}) of 0.3 mm/s with a 3600, 1200, 120, 12, and 1.2 s pre-sliding duration (T_{ps}). Mean ± SEM, n=4.

Role of Fluid Depressurization During Rotation. | N | exhibited transient increases during compression and torsion, that diminished subsequently when motion was halted. During compression, | N | increased to a peak (data not shown), and then relaxed (Fig. 5.9A) with a time constant ($t_{1/2}$) of 27±1 s, achieving an N_{eq} of 3.2±0.2 N. During subsequent torsion at the relatively fast v_{eff} of 3 mm/s (Fig. 5.9B), | N | was cyclical and attained maxima at approximately 180°, 540°, and 900° and minima of approximately the initial value at 360° and 720°. Consistent with the findings noted above, just after the start of rotation, | τ | and thus μ, peaked (see insets of Fig. 5.9CD), and then diminished to values that varied periodically but were approximately at a steady-state by the second revolution (360-720°). Also consistent with the above findings, μ_{static} = 0.27±0.06 (shown in Fig. 5.9E inset) was similar to $\mu_{static,\ Neq}$ = 0.33±0.08 (since | N | immediately after the start of rotation was essentially identical to | N_{eq} |), and $<\mu_{kinetic}>$ = 0.025±0.003 was less than $<\mu_{kinetic,\ Neq}>$ = 0.057±0.010 (since | N | during rotation from 360° to 720° was greater than | N_{eq} |). Then, from the maxima in | N | at 900°, | N | relaxed (Fig. 5.9E) with a time constant ($t_{1/2}$) of 17±1 s, achieving an N_{eq} of 3.0±0.2 N. The normalized time-dependence of relaxation (Fig. 5.9E, right axis) appeared similar qualitatively to the time-dependent relaxation after the initial ramp compression (Fig. 5.9A).

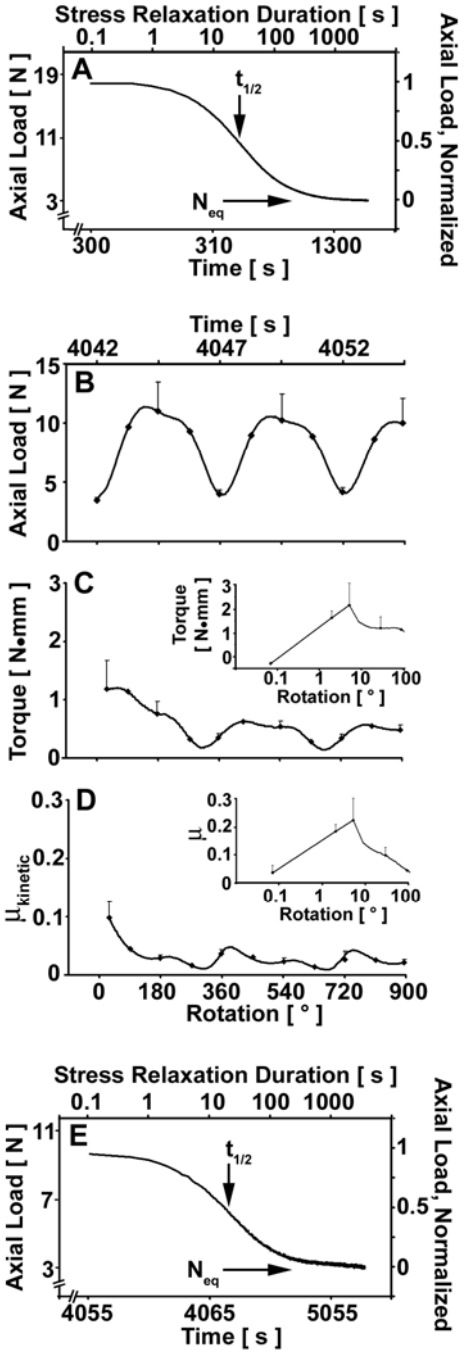

Figure 5.9: Axial load measurements following 18% compression ($1-\Lambda_Z$) (**A**) versus time and stress relaxation duration (T_{sr}). Axial load (**B**) and torque (with log scale insets) (**C**) measurements, and resulting friction coefficient μ (with log scale insets) (**D**), versus rotation at $1-\Lambda_Z$ = 18% after T_{sr} = 60 min, at an effective sliding velocity (v_{eff}) of 3 mm/s with a 120 s pre-sliding duration (T_{ps}), in a test bath of SF. Axial load measurements following rotation (**E**) versus time and T_{sr}. Mean ± SEM, n=4.

5.5 Discussion

The results described here indicate the annulus-on-disk rotational test configuration may be useful for elucidating boundary lubrication at an articular cartilage-on-cartilage interface. At v_{eff} = 0.3 mm/s and 1-Λ_Z = 18%, μ_{static} and $<\mu_{kinetic}>$ varied with T_{sr} in PBS, increasing to peak and approximately steady values of 0.25 and 0.096, respectively. In SF, μ_{static} remained relatively constant at 0.11, while $<\mu_{kinetic}>$ varied with T_{sr}, increasing to a peak value of 0.035. After T_{sr} = 60 min and initial fluid depressurization, in both PBS and SF, $\mu_{static, Neq}$ was approximately equal to μ_{static} (Fig. 5.4). Also, at T_{sr} = 60 min, slow v_{eff} (0.1, 0.3 and 1 mm/s), and a range of compression levels (1-Λ_Z = 18% and 24%), $<\mu_{kinetic, Neq}>$ was steady at 0.093 in PBS and 0.018 in SF (Fig. 5.6). At various T_{ps} (1-3600 s) between the initial fluid depressurization (T_{sr} = 60 min) and start of torsion, with v_{eff} = 0.3 mm/s and 1-Λ_Z = 18%, $<\mu_{kinetic}>$ and $<\mu_{kinetic, Neq}>$ were steady at 0.054 and 0.079 in PBS, and lower at 0.012 and 0.019 in SF, respectively, while $\mu_{static, Neq}$ (which was similar to $\mu_{static,}$) increased with T_{ps}, reaching peak values of 0.43 in PBS, and 0.19 in SF (Fig. 5.8). Collectively these results indicate a boundary lubrication mode is operational at a depressurized articular cartilage-on-cartilage interface with v_{eff} = 0.3 mm/s and 1-Λ_Z = 18% for the annular geometry used here, since $<\mu_{kinetic}>$ was relatively invariant with v_{eff} and 1-Λ_Z, a defining feature of boundary lubrication [28]. The results also indicate SF acts as a boundary lubricant for apposing articular cartilage surfaces.

The use of fresh osteochondral fragments in the annulus-on-disk rotational test configuration required attention to several experimental and theoretical issues.

Samples having a relatively plane cartilage surface, perpendicular to the rotational axis, were verified during test setup by the small axial distance (<0.1 mm, or 4% of the thickness of the apposed articular cartilage) between the initial and final points of contact between the annulus and core (as assessed by $|N|$ during one complete revolution). Cartilage consolidation has been measured to be ~7% *in vivo* by comparison of MRI scans taken before and shortly after various types of exercise [12]. Levels of compression slightly higher than these physiological were used here to ensure full and consistent contact. The resulting tissue surface conformation, due to the depth-varying intrinsic material properties of articular cartilage [8, 38], may have circumvented the need for a gimbaled joint, which is desirable when testing synthetic surfaces [9] to avoid the generation of a fluid wedge. The consistency of friction coefficients calculated from tests over a range of compression amplitudes (12-24%) suggest that the values at the 18% levels of $1-\Lambda_Z$ are physiologically relevant. Potential directional effects on τ measurements were accounted for by averaging the + and – test revolutions and resulted in moderately low variability in μ, both within (coefficient of variation (CV) 14 - 21%) and between animals (CV 19 - 30%), at $1-\Lambda_z$ = 18%, T_{sr} = 60 min, v_{eff} = 0.3 mm/s, and T_{ps} = 120 s. Therefore, with attention to test sample preparation, and subsequent characterization of friction properties, fresh osteochondral samples from non-apposing locations within the synovial joint, can be tested *in vitro* to analyze boundary lubrication at articulating cartilage surfaces.

The cyclical nature of the $|N|$ during rotation (after initial fluid depressurization following axial compression), and the effects of rotation on $|\tau|$

appeared to be explained predominantly by fluid pressurization during rotation, based on experimental and theoretical considerations. Indeed, a similar velocity dependent normal stress was observed when articular cartilage was rotated against a steel surface [45], suggesting that the effect was not due to the fact that both apposed surfaces were articular cartilage. The authors proposed that this effect resulted from steady flow of fluid through the porous permeable solid matrix of cartilage, and possibly from the charged nature of the tissue matrix. In the present study, when rotation was halted, $|N|$ relaxed to $|N_{eq}|$ with a time constant characteristic of fluid depressurization (Fig. 5.9AE). Indeed, the extent of fluid pressurization may be represented by the difference between values of σ_{peak} and σ_{eq}. During this time, $|\tau|$ oscillated, but with an average value during the second test revolution that was virtually unaffected (verified by the ratio of $\tau_{360\text{-}720^\circ}$ to τ_{360° being 95±10% (mean±SD)). This is further supported by $<\mu_{kinetic,\ Neq}>$ being unaffected by v_{eff} at higher compression ($1\text{-}\Lambda_Z$ =18%, used in most experiments, and 24%, Fig. 5.6C), and $<\mu_{kinetic}>$ generally decreasing with increasing v_{eff} (Fig. 5.6B) and consistently being less than the $<\mu_{kinetic,\ Neq}>$ in all test protocols and lubricants (Fig. 5.6, 5.8). Therefore, $<\mu_{kinetic,\ Neq}>$ is an appropriate measure of the frictional response of articular cartilage, minimizing the effects of fluid pressurization, under boundary lubricating conditions, especially for tests at the lower effective sliding velocities.

The results obtained here are consistent with and extend the earlier studies of Davis [9, 10] and Malcom and Fung [16, 29]. In Davis's studies, bovine SF lubricated planed nasal septal cartilage surfaces better than Gey's balanced salt solution at various compressive loads (0.1-0.3MPa) and v_{eff} (0.5-2.5 mm/s). A direct comparison

of μ values is difficult due to the different cartilaginous tissue tested, and the duration allowed to reach equilibrium, nonetheless, $\mu \sim 0.025$ in SF at $v_{eff} = 1$ mm/s is similar to the $<\mu_{kinetic,\ Neq}> = 0.022$ reported here (Fig. 5.6C). Although the effect of fluid pressurization was not characterized, the maintenance of SF's lubricating ability after hyaluronidase treatment implied a boundary mode lubrication was dominant. Davis ultimately reported inconsistencies with repeated testing of the same lubricant and abandoned the use of septal cartilage. This may have resulted from planed septal cartilage surfaces lacking specialized properties of articular cartilage and articular cartilage surfaces, where interactions with lubricant molecules in SF may occur. The relatively low modulus near the surface of articular cartilage may have facilitated the conformation of apposing surfaces [38]. In the studies of Malcom and Fung, SF also lubricated better than PBS under various static loads (0.05 – 5 MPa) at $v_{eff} = \sim 4$ mm/s after creep. The time dependence of $<\mu_{kinetic}>$ at an articular cartilage-on-cartilage interface during creep, rather than stress relaxation (Fig. 5.4), was demonstrated. Malcom and Fung reported $<\mu_{kinetic}> = 0.005 \pm 0.001$ in SF vs. $<\mu_{kinetic}> = 0.010 \pm 0.002$ in PBS (mean±SD) at ~0.1 MPa, with a relative insensitivity of shear friction and therefore $<\mu_{kinetic}>$, to shearing velocity (v_{eff}), over the range presented here (Fig. 5.6). This supports the assertion that the annulus-on-disk rotational configuration is amenable to boundary lubrication of articular cartilage as well. The effect of T_{ps} on μ_{static}, but not $<\mu_{kinetic}>$, was also observed, as in the present study (Fig. 5.8), with μ_{static} ranging from ~0.01 – 0.1 in PBS, and 0.005 – 0.015 in SF, for $T_{ps} = 0 – 8$ min at $v_{eff} = \sim 4$ mm/s. Direct comparison of μ values is again difficult due to differences in loading protocols, and the duration of rotation and fluid depressurization. The values for

$<\mu_{kinetic}>$ reported by Malcom and Fung are approximately ten-fold less than those reported here at similar test parameters, which may be due to continuous rotation during the relatively short time allowed for creep (12 min), since the shear force was shown to increase with time and continuous rotation may 'align' boundary lubricating molecules at the surface. Therefore, the lubrication test configuration developed here is a modified version of that developed by Davis and by Malcom and Fung, with expanded test protocols and characterization.

The effect of fluid pressurization within cartilage on μ is consistent with and extends several studies as well. Wang et al. [45] observed that the normal stress under a prescribed infinitesimal compressive strain increased with increasing sliding velocity, similar to that found in the present study (Fig. 5.5, and in pilot studies with an articular cartilage-polysulfone interface, data not shown), using a plate on plate geometry within a rotational friction apparatus. Krishnan et al. [24] demonstrated a negative correlation between the temporal variation of the effective friction coefficient (μ_{Eff}) of cartilage with the interstitial load support using a reciprocating friction apparatus (v = 1 mm/s) articulating cartilage against glass. Using previously frozen samples, PBS as a test lubricant, and a prescribed load of 4.5N (σ = 0.16 MPa for the sample geometry), μ_{Eff} = ~0.25 was reported after fluid depressurization, more than double compared to the values reported for the corresponding $<\mu_{kinetic}>$ and $<\mu_{kinetic,\ Neq}>$ at v_{eff} = 1mm/s, $1-\Lambda_Z$ = 24% (Fig. 5.6BC). However, in a subsequent study using fresh cartilage samples and the same friction apparatus to assess the role of the superficial zone of articular cartilage when articulated against glass, lower values for μ_{Eff} = ~0.15 after fluid depressurization were reported [23], which are in agreement

with values reported for fresh samples here (μ was not determined in SF in either of these studies). The diminished effect of T_{sr}, and hence fluid depressurization, on the frictional properties of articular cartilage in SF compared to PBS (Fig. 5.4) may be indicative of lubricant molecules interacting with the articular cartilage surface and modulating the frictional response. The time dependence of the friction properties of cartilage has also been observed in a reciprocating motion friction test using cartilage-on-metal contacts, although the absolute values of μ were much greater [14].

The boundary lubricating ability of SF demonstrated here is consistent with several other studies using various friction apparati and test surfaces. Jones originally measured μ of cartilage against cartilage, at very slow rubbing speeds using a horse stifle joint, to be 0.02 in SF [22]. Charnley repeated Jones' experiments using a similar apparatus, and found very low $\mu = 0.005 – 0.024$ [7]. However, other studies of that era indicated that SF had very little lubricating ability between non-cartilaginous surfaces [22, 44]. Linn et al. [28] reported similarly low levels of $\mu = 0.004$ using bovine SF in excised dog ankle joints using an arthrotripsometer. Using a gimbaled annulus-on-disk rotational test configuration, Davis et al. showed bovine SF enhanced boundary lubrication between specific synthetic surfaces, latex on glass, resulting in a $\mu = \sim 0.021$ [10]. More recently, Jay et al. reported healthy bovine and human synovial fluid to have a $\mu = 0.019\text{-}0.028$ [18, 19] and $\mu = \sim 0.025$ [21], respectively, under boundary lubricating conditions. Even though a wide range of μ values are reported in tests using intact joints, likely due to the complex articular cartilage-on-cartilage interaction, the historical values obtained by Jones in the stifle joint and the upper

limit of Charnley's are consistent with those obtained here μ~0.02 (Fig. 5.6). It remains unclear if the physiological molecular structure, and interactions, of boundary lubricants between articular cartilage surfaces are recapitulated with asymmetric synthetic test surfaces, such as latex and glass [10]. Nevertheless, the agreement with μ values obtained here using articular surfaces in a similar test configuration suggests specific synthetic surfaces are useful for studying putative physiological boundary lubricants as well.

The dependency of $\mu_{static,\ Neq}$ on T_{ps}, and other test parameters, is consistent with and extends studies by Forster et al. [13]. They demonstrated the stationary loading time dependence of the start up friction coefficient, with μ values in bovine SF at an articular cartilage-on-cartilage interface ranging from ~0.02 to 0.25 with increasing loading time from 5 s to 45 min under a mixed lubrication regime (σ=0.5-4 MPa and v = 4 mm/s) using a sliding friction machine. Although fluid pressure effects may have been present immediately after start up due to the linear nature of the system with the cartilage plug sliding along a previously unloaded and therefore fully hydrated cartilage surface, the start up friction coefficients values are consistent with $\mu_{static,\ Neq}$ found here at a slower v_{eff} = 0.3 mm/s ranging from 0.02 to 0.19 (Fig. 5.8A). Interestingly, they also demonstrated the ability of bovine SF to reduce start up friction at a cartilage-on-cartilage interface was lost at a cartilage-on-metal interface. Finally, the inverse dependence of $\mu_{static,\ Neq}$ on $1-\Lambda_Z$ in both lubricants (Fig. 5.6A) may be indicative of restrained surface tissue shear at start-up, and potentially chondrocyte protection from wear and mechanical disturbances *in vivo*.

The paradigm of several operational lubrication modes during cartilage articulation within the synovial joint [47] has long been generally accepted. Recently, the natural lubricant constituents in SF such as proteins, lipids, and hyaluronic acid were proposed to act synergistically in the synovial joint through adaptive multimode lubrication [35]. Dowson stated that a full appreciation of the tribological performance of joints can be achieved only when it is known whether the mode of lubrication is fluid film, boundary or mixed, and, that previous attempts to ascribe a single mode of lubrication to synovial joints have undoubtedly delayed the emergence of a satisfactory overall picture of the performance of natures bearing [11]. Although the various friction properties of articular cartilage characterized in this study have been previously demonstrated, the wide range of reported μ values indicates the need for careful characterization of the test setup, sample surface, preparation and storage, and resulting measurements in control type lubricants to identify the operating lubrication mode. Only then can quantitative, mechanistic statements be made about the boundary lubricating properties of cartilage within synovial joints. Therefore, this test configuration, particularly with parameters of v_{eff} = 0.3 mm/s and 1-Λ_Z = 18%, after fluid depressurization, is useful for defining the lubrication properties of putative fluid lubricants; it may also allow elucidation of the components of synovial fluid that function, independently, additively, or synergistically, as boundary lubricants [17, 20, 28, 39, 40, 43] through potentially specific interactions with native articular cartilage surfaces.

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CHAPTER 6

BOUNDARY LUBRICATION OF ARTICULAR CARTILAGE: ROLE OF SYNOVIAL FLUID CONSTITUENTS

6.1 Abstract

Objective. The objective of this study was to determine if synovial fluid (SF) constituents: hyaluronan (HA), proteoglycan 4 (PRG4), and surface active phospholipids (SAPL), contribute to boundary lubrication, either independently or additively, at an articular cartilage-cartilage interface.

Methods. Cartilage boundary lubrication tests were performed with fresh bovine osteochondral samples. Tests were performed in graded concentrations of SF, HA, and PRG4 alone; a physiological concentration of SAPL; and various combinations of HA, PRG4, and SAPL at physiological concentrations. Static, $\mu_{static,\ Neq}$, and kinetic, $<\mu_{kinetic,\ Neq}>$, friction coefficients were calculated.

Results. Normal SF functioned as an effective boundary lubricant, $<\mu_{kinetic, Neq}>$ = 0.025, even with a 3-fold decrease in constituent concentration, $<\mu_{kinetic, Neq}>$ = 0.029. Both HA and PRG4 contributed independently to a low μ in a dose-dependent manner. Values of $<\mu_{kinetic, Neq}>$ decreased to from ~ 0.24 in PBS to 0.12 in 3300 μg/ml HA, and 0.11 in 450 μg/ml PRG4. HA and PRG4 in combination lowered μ further at these concentrations, attaining a value of $<\mu_{kinetic, Neq}>$ = 0.066. SAPL at 200 μg/ml did not significantly lower μ, either independently or in combination with HA and PRG4.

Conclusion. The results described here indicate SF constituents contribute, individually and in combination, both at physiological and pathophysiological concentrations, to the boundary lubrication of opposing articular cartilage surfaces. These results provide insight into the nature of the boundary lubrication of articular cartilage with SF and its constituents, which is relevant to both the homeostatic maintenance of healthy joints as well as the pathogenic processes in arthritic disease.

6.2 Introduction

Articular cartilage is the lubricious, load bearing tissue at the end of long bones in synovial joints that normally facilitates low-friction and low-wear articulation [64]. When healthy, it provides low-friction properties to the synovial joint through a combination of lubrication mechanisms [3]. Pressurized fluid, within the tissue and between the surfaces, such as in a fluid film, can bear significant portions of the load [2]. Lubricant molecules, within a surface layer or film at the articular surface, mediate load bearing surface-to-surface contact in boundary lubrication [44]. This mode of lubrication has been proposed to be important for the protection and maintenance of articular surfaces since the opposing cartilage layers within the joint make contact over ~10% of the total area, where most of the friction may occur [45]. Synovial fluid (SF) contains the molecules hyaluronan (HA) [48], proteoglycan 4 (PRG4) [60], and surface active phospholipids (SAPL) [56], that interact with and adsorb to the articular surface. Such molecules are all ideally positioned to contribute to boundary lubrication.

SF, as well as HA, PRG4, and SAPL, have each demonstrated boundary lubricating ability at various test interfaces. SF was recently shown to function as an effective boundary lubricant at a depressurized articular cartilage-cartilage interface using an annulus on disk configuration (Chapter 5). These results were consistent with and extended several previous studies using native cartilage surfaces [9, 35], and synthetic surfaces with a similar test configuration [12, 27, 28, 32]. The lubricating ability of HA has been assessed at cartilage-cartilage [5, 7, 20, 36, 39, 41, 52, 66],

cartilage-steel [46] and cartilage-glass interfaces [20, 38, 58], as well as a latex-glass interface under boundary lubrication conditions [28, 31, 32]. The conclusions from these studies regarding the boundary lubricating ability of HA were somewhat conflicting, possibly due to the different test surfaces and test configurations used, and the various resulting operative modes of lubrication. Conversely, PRG4 proteins [23], which are synthesized and secreted by cells lining the synovial cavity [26, 53, 60, 62], have consistently demonstrated boundary lubricating ability at both cartilage-glass [58, 65, 67], and latex-glass interfaces [24, 26, 28-31, 33]. Studies examining the lubricating ability of SAPL at a cartilage-cartilage interface (in combination with HA) [46], a cartilage-steel interface [46, 49], as well as a latex-glass interface under boundary lubrication conditions [27], suggest SAPL may also possess boundary lubricating ability. Collectively, these studies suggest HA, PRG4, and SAPL each may contribute to the boundary lubricating ability of SF at a cartilage-cartilage interface.

Accordingly, the boundary lubricating ability of SF may be diminished in injury and disease due to the alteration in concentration of HA, PRG4, and SAPL. The concentration of HA in human SF ranges from 1-4 mg/ml in healthy individuals [4, 10, 43, 69], and decreases after effusive joint injury [1] and in arthritic disease to ~0.1-1.3 mg/ml [11, 43]. The concentration of PRG4 in human SF ranges from 52-350 μg/ml post-mortem, and 276-762 μg/ml in SF obtained from patients undergoing arthrocentesis procedures [57]. Conversely, using a rabbit knee injury model, the concentration of PRG4 in SF decreased from 280 μg/ml to 20-100 μg/ml, 3 weeks after injury [13]. The majority of the lipids in human SF are phospholipids, whose concentration range from ~0.1-0.2 mg/ml in normal individuals, increases in

osteoarthritis to ~0.2-0.3 mg/ml [43], and can decrease following traumatic injury to ~0.02-0.08 mg/ml [51]. While most phospholipids are surface active, dipalmitoyl-phosphatidylcholine (DPPC) is particularly so, and is the most abundant form present in SF at ~45% [22, 56].

The governing hypothesis of this study was that SF constituents contribute to the boundary lubrication of articular cartilage. The specific objective of this study was to determine if SF constituents: HA, PRG4, and SAPL, contribute to boundary lubrication, either independently or additively, at an articular cartilage-cartilage interface. To achieve this objective, the effect of graded concentrations of SF on cartilage boundary lubrication was first determined. Then, the independent effects of graded concentrations of HA, and PRG4, and a physiological concentration of SAPL, on cartilage boundary lubrication were determined. Lastly, the additive effect of physiological concentrations of HA, PRG4, and SAPL in various combinations, on cartilage boundary lubrication was determined.

6.3 Materials and Methods

Materials. Materials for lubrication testing were obtained as described previously (Chapter 5). In addition, high molecular weight sodium hyaluronate (HA), (SupARTZ®, 10 mg/ml, 620 - 1,170 kDa) was obtained from Seikagaku (Tokyo, Japan).

Lubricant Preparation & Characterization. The concentration of HA, PRG4, and phospholipids in the test lubricants was determined by the carbazole reaction for uronic acid [8], ELISA [59], and phospholipid assay using Phospholipids B Standard Solution and Color Reagent (Wako Chemicals, Richmond, VA) [43], respectively.

HA. The concentration of HA in the SupARTZ® HA stock solution was confirmed prior to storage at -20 ºC.

PRG4. PRG4 was prepared from ~300 cartilage disks (6 mm diameter and ~0.3 mm thick, including the articular surface) harvested from 6 immature bovine stifle joints. Cartilage disks were incubated for 6-15 days in DMEM with 0.01% BSA, 25 μg/ml ascorbic acid and 10 ng/ml rhTGF-β1 (PeproTech, Inc. Rocky Hill, NJ). Culture medium was changed every three days and collected for processing. To purify PRG4, the conditioned culture medium was saved, pooled, and fractionated by anion-exchange chromatography with DEAE Sepharose^{TM} gel essentially as described previously [60]. Briefly, the DEAE Sepharose^{TM} was packed and equilibrated with 0.15M NaCl, 0.005M EDTA, and 0.05M sodium acetate, pH 6. The 0.3-0.6M NaCl eluate, in the same buffer, was collected, concentrated with a Centricon® Plus 100

kDa molecular weight (MW) cutoff filter, and then quantified by ELISA [59] using mAb 3A4 (a gift from Dr. Bruce Caterson) [61], prior to storage at -20 ºC. Control studies indicated the DEAE buffer used for PRG4 purification did not alter boundary lubricating ability since SF samples (described below) retained lubricating ability after dialysis against the buffer.

The size distribution of immunoreactive PRG4 was characterized by Western Blot using mAb 3A4 after electrophoresis on a 4-20% SDS-polyacrylamide gel, and transfer to a PVDF membrane. A single immunoreactive band at ~345 kDa was visualized by ECL-Plus detection and digital scanning with a STORM 840 Imaging System (Molecular Dynamics, Fairfield, CT). The concentration of HA (determined from uronic acid concentration) and phospholipids in the PRG4 preparation (at 450 μg/ml) was 30 μg/ml and <0.5 μg/ml, respectively.

SAPL. A 10X solution of DPPC at 2000 μg/ml was sonicated (Sonics & Materials, Danbury, CT) [21, 27] in phosphate buffered saline (PBS) for 15 min to solubolize the DPPC. The concentration of SAPL was confirmed prior to storage at -20 ºC.

SF. Normal bovine SF pooled from 5 animals was obtained as described previously (Chapter 5), and clarified by centrifugation (10,000 g for 60 min at 4 ºC) prior to storage at -80 ºC. The concentration of HA and phospholipid was ~1000 μg/ml, and ~100 μg/ml, respectively. The concentration of the major immunoreactive PRG4 band at ~345 kDa, visualized by Western Blot (described above) after hyaluronidase treatment, was calculated to be ~ 450 μg/ml by quantitative comparison

to a similar MW band of a known amount of purified bovine PRG4 [60], as determined by ELISA [59] (describe above).

Sample Preparation. Fresh osteochondral samples (n=40) were prepared for friction testing from the patellofemoral groove of 10 skeletally bovine stifle joints, as described previously (Chapter 5). Briefly, each sample consisted of an osteochondral core (radius = 6 mm), and an opposed osteochondral annulus (outer radius, R_o = 3.2 mm, and inner radius, R_i = 1.5 mm), both with central holes (radius = 0.5 mm) drilled down into and exiting the bone to facilitate fluid depressurization. In addition, samples were rinsed vigorously overnight in ~40 ml of PBS to deplete the articular surface of residual SF. (Pilot studies confirmed the glycosaminoglycan content within the articular cartilage [14] of rinsed samples was similar, within 1%, to that of non-rinsed samples (n=4, p=0.83)). Samples were bathed in ~0.5 ml test lubricant, completely immersing the cartilage, at 4 °C for 24 hr prior to lubrication testing.

Lubrication Test. Cartilage boundary lubrication tests (Fig. 6.1) were performed essentially as described previously (Chapter 5). Briefly, samples of articulating cartilage were preconditioned by compressing at a constant rate of 0.002 mm/s to a compression level ($1-\Lambda_Z$) = 18% of the total cartilage thickness, rotated +2 revolutions and then -2 revolutions at an effective velocity (v_{eff}) = 3 mm/s, and then unloaded to 0%. This sequence was then repeated twice more. Samples were then tested by first compressing to $1-\Lambda_Z$ = 18% and allowing a 60 min stress relaxation duration (T_{sr}) for fluid depressurization. Then, samples were rotated +2 revolutions and then -2

revolutions at v_{eff} = 0.3 mm/s, with pre-spin durations (T_{ps}) of 1200, 120, 12, and 1.2 s. The test sequence was then repeated in the opposite direction of rotation.

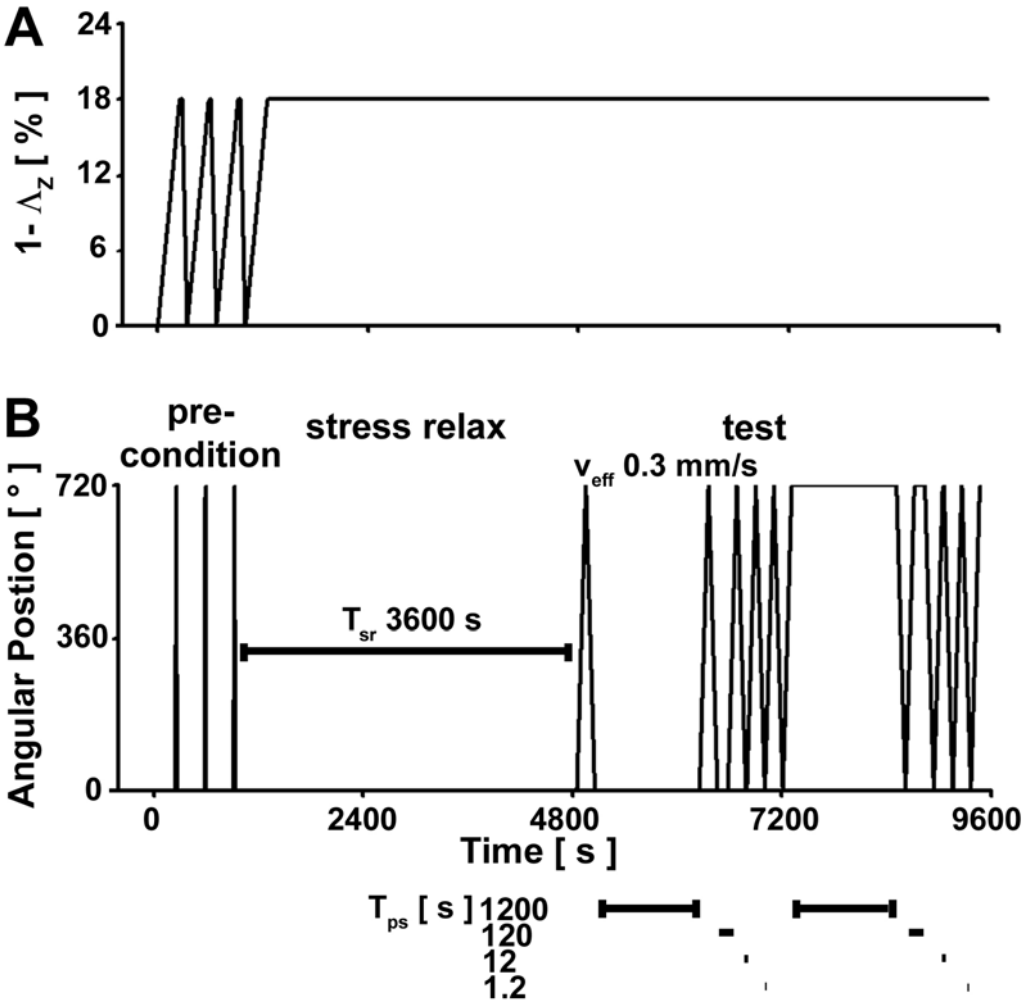

Figure 6.1: Boundary lubrication test protocol. Osteochondral annulus and core samples were compressed axially by $1\text{-}\Lambda_Z = 18\%$ of the total cartilage thickness (**A**) A rotational test protocol with pre-conditioning, a stress relaxation duration $T_{sr} = 3600$ s, and effective sliding velocity $v_{eff} = 0.3$ mm/s was then used to determine the effects of test lubricants and pre-sliding duration T_{ps} (1200 – 1.2 s) on the boundary lubrication of articular cartilage (**B**).

Experimental Design. To determine if HA, PRG4, and SAPL contribute to cartilage boundary lubrication, either independently or additively, test lubricants were prepared in PBS. In all experiments, PBS and SF, served as negative and positive controls, on the first and last day of lubrication testing, respectively.

Graded concentrations of SF. To determine the effect of graded concentrations of SF on cartilage boundary lubrication, tests were performed in: PBS, 3.3% SF, 10% SF, 33% SF, and then SF.

SF constituents alone. To determine the independent effects of graded concentrations of HA, and PRG4, and a physiological concentration of SAPL, on cartilage boundary lubrication, 3 sequences of tests were performed. For HA, tests were performed in: PBS, 110 μg/ml HA, 1100 μg/ml HA, 3300 μg/ml HA, and then SF. For PRG4, tests were performed in: PBS, 4.5 μg/ml PRG4, 45 μg/ml PRG4, 450 μg/ml PRG4, and then SF. For SAPL, tests were performed in: PBS, 200 μg/ml SAPL, and then SF.

SF constituents in combination. To determine the additive effect of physiological concentrations of HA (3300 μg/ml), PRG4 (450 μg/ml), and SAPL (200 μg/ml) in various combinations, on cartilage boundary lubrication, tests were performed in: PBS, HA or PRG4, HA+PRG4, HA+PRG4+SAPL, and then SF.

Data Analysis. To evaluate the boundary lubrication properties of test lubricants, two friction coefficients (μ) were determined, as described previously (Chapter 5). These parameters were calculated from the expression $\mu = \tau / (R_{eff} \bullet N_{eq})$ (where τ is torque,

N_{eq} is the equilibrium axial load after the 60 min stress relaxation duration, and R_{eff} is the effective radius of the annulus sample [42]). Briefly, a static friction coefficient, $\mu_{static, Neq}$, was calculated using the peak $|\tau|$, measured just after (within 10°) the start of rotation, and the equilibrium axial load at the end of the 60 min stress relaxation period, N_{eq}. A kinetic friction coefficient, $<\mu_{kinetic, Neq}>$, was calculated using the $|\tau|$ averaged during the second complete revolution of the test sample, and N_{eq}. $\mu_{static, Neq}$ and $<\mu_{kinetic, Neq}>$ were then averaged for the + and – revolutions in each test. The normal stress (σ), in units of MPa, was also calculated, as described previously (Chapter 5).

Data are presented as mean±SEM. The effects of test lubricant and T_{ps} (as a repeated factor) on the friction coefficients were assessed by ANOVA. In all test lubricants, $<\mu_{kinetic, Neq}>$ varied slightly with T_{ps}, with values at T_{ps} = 1.2 s being on average within 9±11%, or 0.009±0.015 (mean±SD), of values at T_{ps} = 1200 s (which ranged from ~ 0.02–0.3) ($p<0.001$). Therefore, for brevity and clarity, $<\mu_{kinetic, Neq}>$ data is presented at T_{ps} = 1.2 s only. Accordingly, the effect of test lubricant on $<\mu_{kinetic, Neq}>$ at T_{ps} = 1.2 s was assessed by ANOVA, with Tukey post hoc testing. Statistical analysis was implemented with Systat 10.2 (Systat Software, Inc., Richmond, CA).

6.4 Results

Lubrication Test Characterization. In all experiments, friction was modulated by test lubricant and T_{ps}. In all test lubricants, $\mu_{static,\ Neq}$ increased with increasing T_{ps}, and appeared to approach $<\mu_{kinetic,\ Neq}>$ asymptotically as T_{ps} decreased from 1200 s toward 0 s (to 1.2 s). Values of $\mu_{static,\ Neq}$ were consistently greatest in PBS, ranging from 0.34±0.06 to 0.58±0.03 (mean±SD) with increasing T_{ps}; values of $\mu_{static,\ Neq}$ were consistently lowest in SF, ranging from 0.037±0.008 to 0.22±0.02, (mean±SD) with increasing T_{ps}. Similarly with $<\mu_{kinetic,\ Neq}>$, values were greatest in PBS, 0.24±0.04, and lowest in SF, 0.028±0.006 (mean±SD). Equilibrium σ values at T_{sr} = 60 min, 0.11±0.02 MPa (mean±SD), were similar in all test lubricants ($p=0.15$).

Effect of graded concentrations of SF. Friction coefficients were reduced by SF in a dose-dependent manner (Fig. 6.2). $\mu_{static,\ Neq}$ varied with test lubricant ($p<0.001$) and T_{ps} ($p<0.001$), with an interaction effect ($p<0.001$) (Fig. 6.2A). Values of $\mu_{static,\ Neq}$ decreased with increasing concentrations of SF at all T_{ps}. At T_{ps} = 120 s, values of $\mu_{static,\ Neq}$ decreased from 0.40±0.03 in PBS, to 0.117±0.006 and 0.120±0.006 in 33% SF and SF, respectively. $<\mu_{kinetic,\ Neq}>$ at T_{ps} = 1.2 s also varied with test lubricant ($p<0.001$) (Fig. 6.2B). Values of $<\mu_{kinetic,\ Neq}>$ decreased significantly with increasing SF, from 0.20±0.02 in PBS to 0.11±0.02 in 10% SF ($p<0.01$), down to 0.029±0.002 ($p<0.01$) and 0.025±0.005 ($p<0.01$) in 33% SF and SF, respectively.

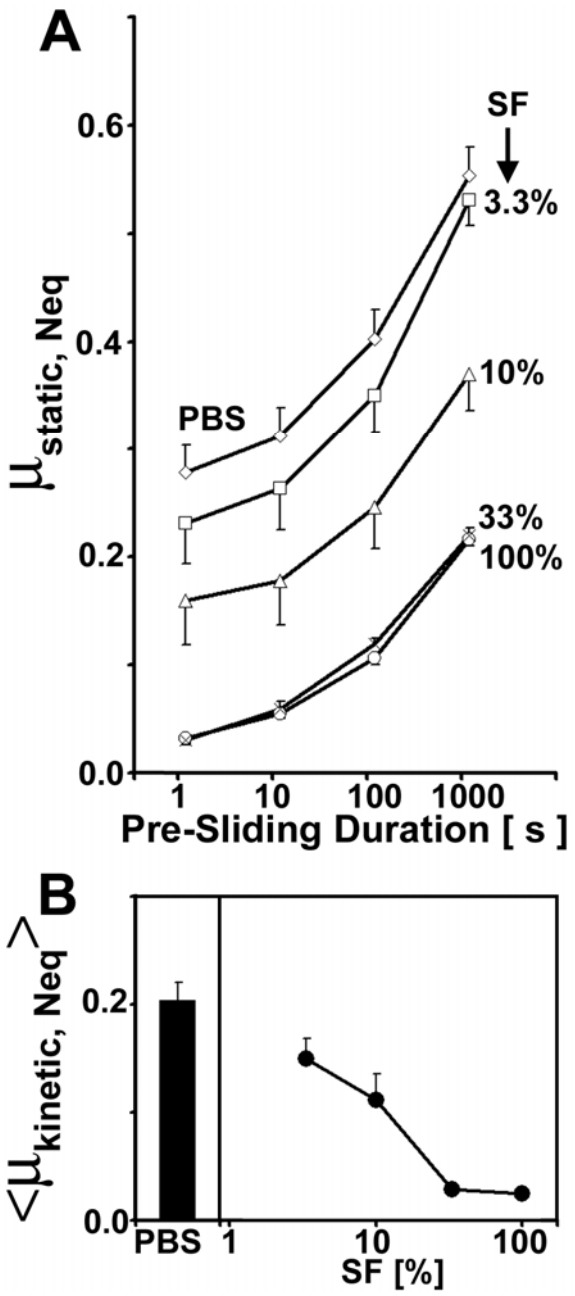

Figure 6.2: Effect of graded concentrations of SF on the boundary lubrication of articular cartilage. Static, $\mu_{static,\ Neq}$ (**A**) and kinetic, $<\mu_{kinetic,\ Neq}>$ at a pre-sliding duration, T_{ps}, of 1.2 s (**B**) friction coefficients in PBS and 3.3 - 100% SF. Mean ± SEM, n=4-8.

Effect of SF constituents alone.

HA. Friction coefficients were reduced by HA in a dose-dependent manner (Fig. 6.3). $\mu_{static,\ Neq}$ varied with test lubricant ($p<0.001$) and T_{ps} ($p<0.001$), with an interaction effect ($p<0.01$) (Fig. 6.3A). Values of $\mu_{static,\ Neq}$ decreased with increasing concentrations of HA at all T_{ps}. At T_{ps} = 120 s, values of $\mu_{static,\ Neq}$ decreased from 0.46±0.02 in PBS to 0.22±0.02 in 3330 μg/ml HA, with SF being the lowest at 0.11±0.01. $<\mu_{kinetic,\ Neq}>$ at T_{ps} = 1.2 s also varied with test lubricant ($p<0.001$) (Fig. 6.3B) Values of $<\mu_{kinetic,\ Neq}>$ decreased significantly with increasing concentrations of HA, from 0.26±0.01 in PBS to 0.21±0.02 in 110 μg/ml HA ($p<0.05$), down to 0.118±0.009 in 3300 μg/ml HA ($p<0.01$), which was greater than 0.031±0.004 in SF ($p<0.01$).

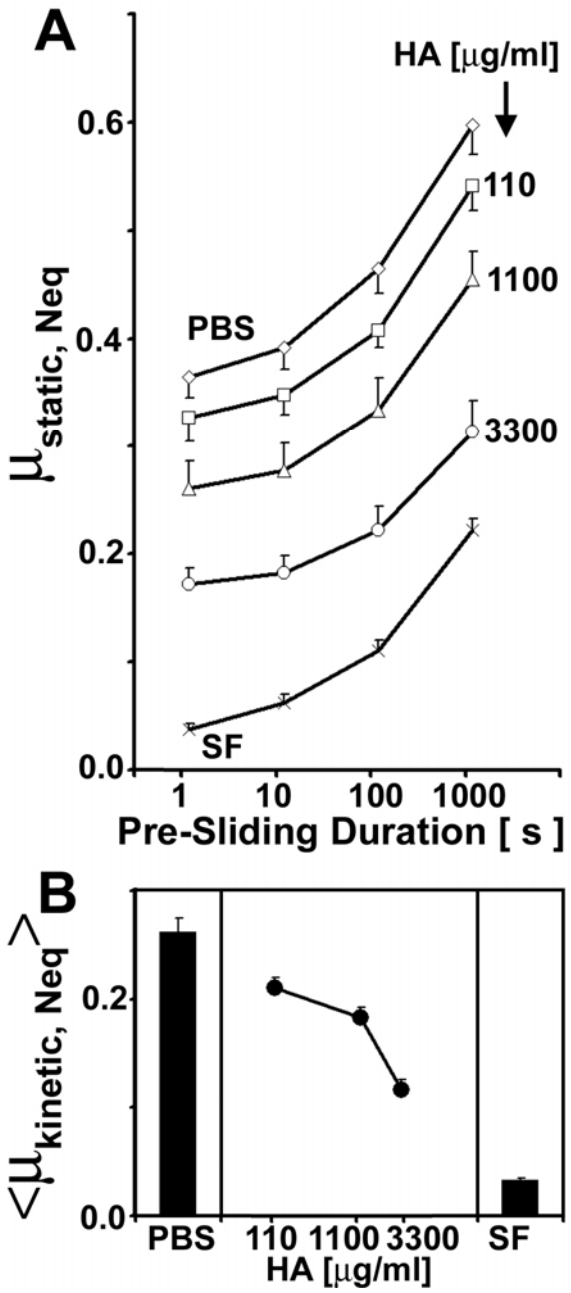

Figure 6.3: Effect of graded concentrations of HA on the boundary lubrication of articular cartilage. Static, $\mu_{static,\ Neq}$ (**A**) and kinetic, $<\mu_{kinetic,\ Neq}>$ at a pre-sliding duration, T_{ps}, of 1.2 s (**B**) friction coefficients in PBS, 110 – 3330 μg/ml HA, and SF. Mean ± SEM, n=4-8.

PRG4. Friction coefficients were reduced by PRG4 in a dose-dependent manner (Fig. 6.4). $\mu_{static,\ Neq}$ varied with test lubricant ($p<0.001$) and T_{ps} ($p<0.001$), with no interaction effect ($p=0.72$) (Fig. 6.4A). Values of $\mu_{static,\ Neq}$ decreased with increasing concentrations of PRG4 at all T_{ps}. At T_{ps} = 120 s, values of $\mu_{static,\ Neq}$ decreased from 0.48±0.02 in PBS to 0.27±0.03 in 450 μg/ml PRG4, with SF being the lowest at 0.12±0.02. $<\mu_{kinetic,\ Neq}>$ at T_{ps} = 1.2 s also varied with test lubricant ($p<0.001$) (Fig. 6.4B). Values of $<\mu_{kinetic,\ Neq}>$ decreased significantly with increasing concentrations of PRG4, from 0.23±0.02 in PBS and 0.20±0.01 in 4.5 μg/ml PRG4, to 0.10±0.02 in 450 μg/ml PRG4 (both $p<0.001$), which was greater than 0.04±0.01 in SF ($p<0.05$).

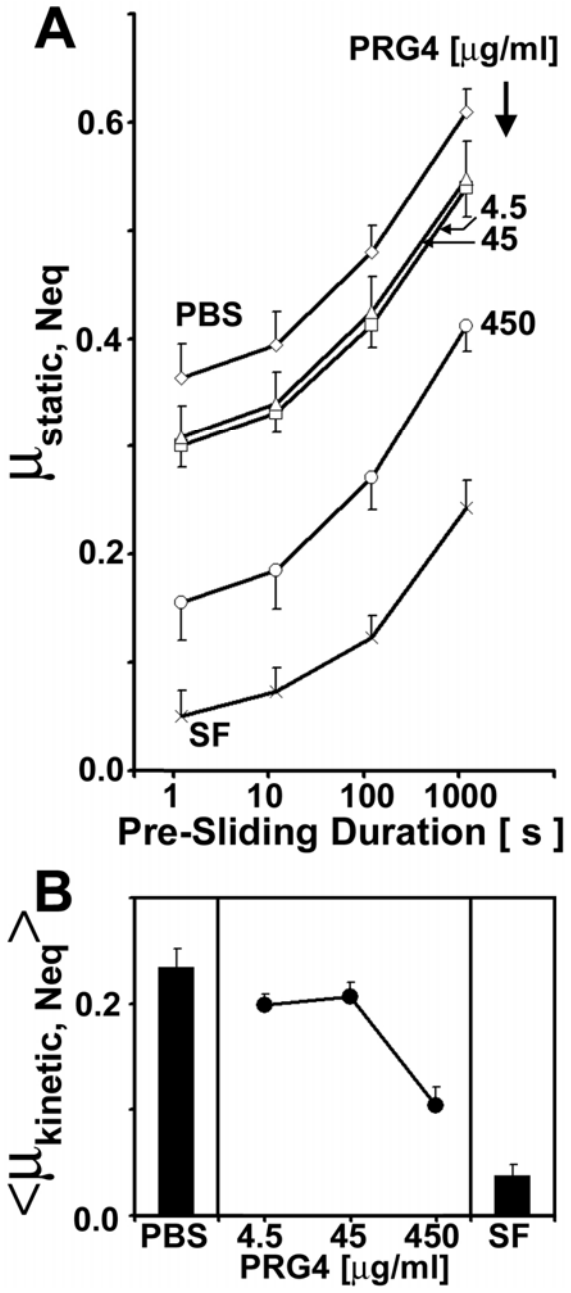

Figure 6.4: Effect of graded concentrations of PRG4 on the boundary lubrication of articular cartilage. Static, $\mu_{static,\ Neq}$ (**A**) and kinetic, $<\mu_{kinetic,\ Neq}>$ at a pre-sliding duration, T_{ps}, of 1.2 s (**B**) friction coefficients in PBS, 4.5 - 450 μg/ml PRG4, and SF. Mean ± SEM, n=8.

SAPL. Friction coefficients were not affected by SAPL (Fig. 6.5). $\mu_{static,\ Neq}$ varied with test lubricant ($p<0.001$) and T_{ps} ($p<0.001$), with an interaction effect ($p<0.001$) (Fig. 6.5A). Values of $\mu_{static,\ Neq}$ appeared to decrease slightly with the addition of SAPL compared to PBS at all T_{ps}. At T_{ps} = 120 s, values of $\mu_{static,\ Neq}$ ranged from 0.39±0.02 in PBS to 0.34±0.02 in 200 μg/ml SAPL, with SF being the lowest at 0.110±0.009. $<\mu_{kinetic,\ Neq}>$ at T_{ps} = 1.2 s also varied with test lubricant ($p<0.001$) (Fig. 6.5B). Values of $<\mu_{kinetic,\ Neq}>$ did not decrease significantly from 0.21±0.02 in PBS to 0.17±0.01 in 200 μg/ml SAPL ($p=0.17$), which was greater than 0.031±0.002 in SF ($p<0.001$).

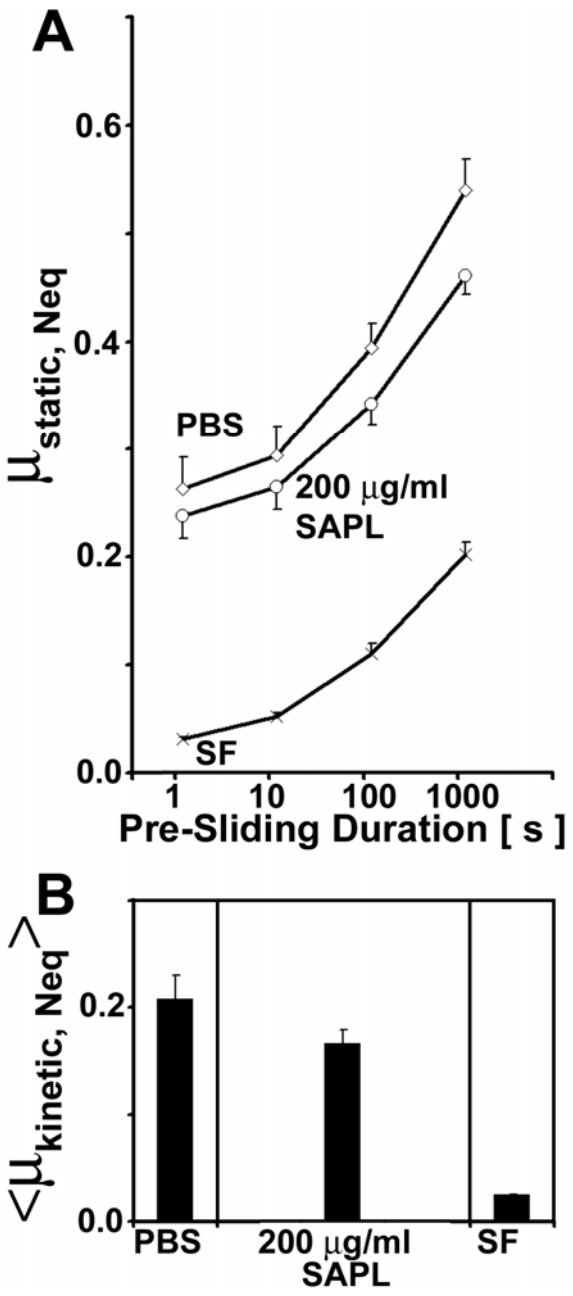

Figure 6.5: Effect of SAPL on the boundary lubrication of articular cartilage. Static, $\mu_{static,\ Neq}$ (**A**) and kinetic, $<\mu_{kinetic,\ Neq}>$ at a pre-sliding duration, T_{ps}, of 1.2 s (**B**) friction coefficients in PBS, 200 μg/ml SAPL, and SF. Mean ± SEM, n=8.

Effect of SF constituents in combination. Friction coefficients were reduced by certain combinations of HA, PRG4, and SAPL at physiological concentrations of 3330 μg/ml, 450 μg/ml, and 200 μg/ml, respectively (Fig. 6.6). $\mu_{static,\ Neq}$ varied with test lubricant ($p<0.001$) and T_{ps} ($p<0.001$), with an interaction effect ($p<0.001$) (Fig. 6.6A). Values of $\mu_{static,\ Neq}$ decreased with the addition of HA and/or PRG4 at all T_{ps}. At T_{ps} = 120 s, values of $\mu_{static,\ Neq}$ decreased from 0.51±0.02 in PBS, to 0.22±0.02 in HA and 0.23±0.02 in PRG4, to 0.18±0.01 in HA+PRG4, with SF being the lowest at 0.130±0.008. $<\mu_{kinetic,\ Neq}>$ at T_{ps} = 1.2 s also varied with test lubricant ($p<0.001$) (Fig. 6.6B). Values of $<\mu_{kinetic,\ Neq}>$ decreased significantly with the addition of HA and/or PRG4, from 0.29±0.01 in PBS to 0.12±0.01 in HA ($p<0.01$) and 0.11±0.01 in PRG4 ($p<0.01$), to 0.066±0.003 in HA+PRG4 ($p<0.05$ and $p<0.01$, respectively), which was greater than 0.024±0.003 in SF ($p<0.05$). The addition of SAPL did not significantly lower $<\mu_{kinetic,\ Neq}>$ (0.062±0.002) from the value in HA+PRG4 ($p=0.99$).

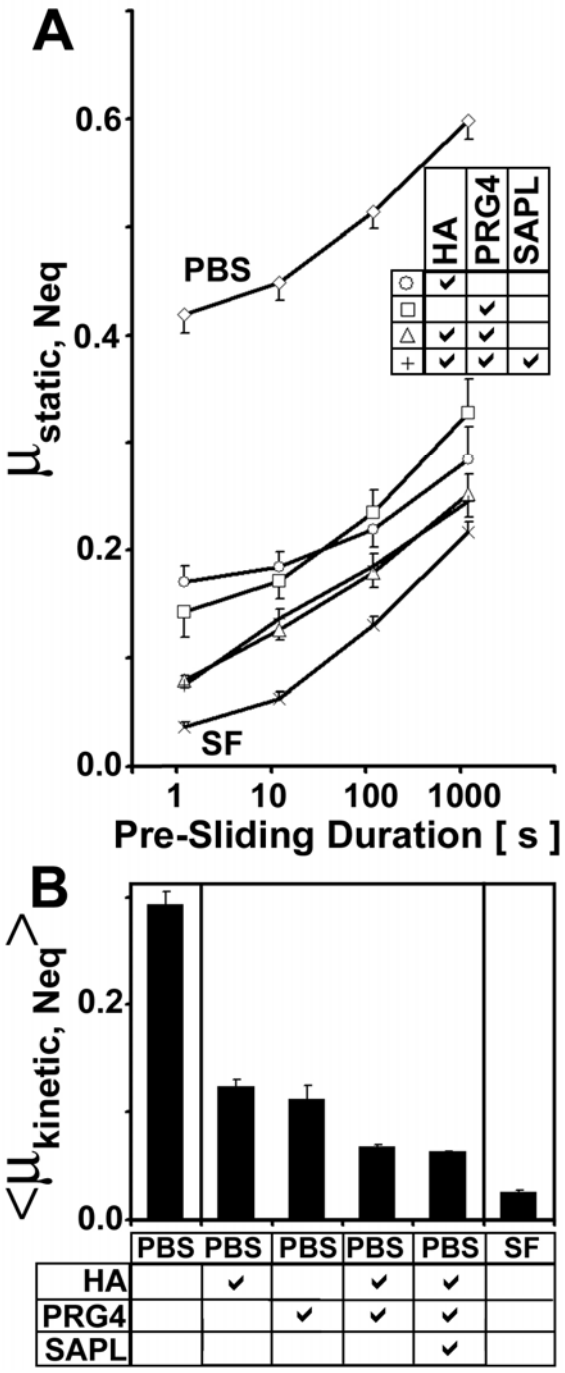

Figure 6.6: Effect of HA, PRG4, and SAPL in combination on the boundary lubrication of articular cartilage. Static, $\mu_{static,\ Neq}$ (**A**) and kinetic, $<\mu_{kinetic,\ Neq}>$ at a pre-sliding duration, T_{ps}, of 1.2 s (**B**) friction coefficients in PBS, PBS + 3330 μg/ml HA, PBS + 450 μg/ml PRG4, PBS + HA + PRG4, PBS + HA + PRG4 + 200 μg/ml SAPL, and SF. Mean ± SEM, n=4-8.

6.5 Discussion

The results described here indicate SF constituents contribute, individually and in combination, both at physiological and pathophysiological concentrations, to the boundary lubrication of opposing articular cartilage surfaces. Normal SF functioned as an effective boundary lubricant at the articular cartilage-cartilage interface tested here, $<\mu_{kinetic,\ Neq}> = 0.025$, even with a 3-fold decrease in constituent concentration, $<\mu_{kinetic,\ Neq}> = 0.029$ (Fig. 6.2B). Both HA, and the PRG4 preparation used here, contributed independently to a low μ in a dose-dependent manner. Values of $<\mu_{kinetic,\ Neq}>$ decreased to from ~ 0.24 in PBS to 0.12 in 3300 μg/ml HA (Fig. 6.3B), and 0.11 in 450 μg/ml PRG4 (Fig. 6.4B). HA and PRG4 in combination lowered μ further at these concentrations, attaining a value of $<\mu_{kinetic,\ Neq}> = 0.066$ (Fig. 6.6B). SAPL at 200 μg/ml did not significantly lower μ, either independently or in combination with HA and PRG4. Collectively, these results suggest SF constituents, PRG4 and HA, synergistically contribute to the effective boundary lubrication of articular cartilage.

The SF constituents used in the present study were representative of those in native SF. SF is composed of HA ranging from 2,000–10,000 kDa [11, 16, 17], PRG4 proteins ranging from ~14-345 kDa [15, 24, 54, 60] (in unknown proportions), and different types of SAPL [51]. HA has been shown to lubricate at a cartilage-cartilage interface on a joint scale equally well at 1,030 and 1,930 kDa [41], and certain other properties of HA do not depend on MW either (in the range of 500-6,000kDa) [19]. Therefore, the use of SupARTZ® HA (average MW 800 kDa) in the present study was reasonable for studying the boundary lubricating ability of HA. The large central

mucin-like domain reported to mediate the boundary lubricating ability of PRG4 [29] is present in the various >~220 kDa forms of PRG4 [33], therefore the use of a ~345 kDa form of PRG4 prepared from conditioned media was reasonable as well. Finally, DPPC is the major component of SAPL in SF [22], and was therefore also an appropriate choice. Similar SF constituents have been used in several previous studies examining their lubricating function [20, 24, 26, 28-31, 33, 36, 49, 58, 65, 67], and future studies may be useful in determining the potential additional friction lowering effects of specific MW and/or forms of SF constituents. Additional studies examining the structure-function relationship of SF constituents contributing to boundary lubrication, both alone and in combination, will provide the framework for the potential complete recapitulation of SF's boundary lubricating ability with a combination of individual constituents. In the present study, the observed friction lowering effect of the constituents used at physiological concentrations, suggests they are at least necessary for the boundary lubrication of articular cartilage. Therefore, these results contribute to the elucidation of physiological boundary lubricants present in SF.

The dose-dependent boundary lubricating abilities of SF, as well as PRG4 individually, are consistent with and extend several previous studies. Swann *et al.* demonstrated a dose-dependent effect of SF at a cartilage-glass interface [65]. Using the test configuration and protocol used in the present study (Chapter 5), SF was previously demonstrated to be an effective boundary lubricant with a similar value of $<\mu_{kinetic,\ Neq}>$ ~ 0.02. In the present study, the values of $<\mu_{kinetic,\ Neq}>$ ~ 0.24 in PBS were considerably higher then those reported previously (Chapter 5), ~ 0.07. As others

have noted [39], this can be attributed to the rinsing of samples in PBS after harvest, to remove residual SF from the articular surface, prior to testing in PBS. The dose-dependent effect of PRG4 is consistent with a previous study by Jay *et al.* [24], using a similar test configuration with a latex-glass interface, where lubrication function occurred at concentrations greater than 200 μg/ml. However, the absolute value of $<\mu_{kinetic,\ Neq}>$ ~ 0.10 in 450 μg/ml PRG4 observed here (Fig. 6.4B) is greater than the range of μ values reported in several other studies by Jay *et al.* [26, 28, 29, 33], ~ 0.047-0.018 in 250-400 μg/ml PRG4. This difference may be attributable to potentially specific interactions of PRG4 with native articular cartilage surfaces. Nevertheless, the results of the present study indicate that PRG4 contributes to the boundary lubrication of articular cartilage, as previously inferred from studies at a latex-glass interface. These contributions were specific, since the HA and SAPL content in the preparation were low, and control proteins (albumins and globulins) at a physiological concentration did not independently lower μ (data not shown).

The significant contribution of HA to the boundary lubrication of apposed articular cartilage surfaces reported here extends previous studies examining the lubricating ability of HA with test protocols and/or configurations where a boundary mode of lubrication was dominant. Bell *et al.* [5] demonstrated Arthrease™, a fermentation derived sodium hyaluronate with a MW of 3,000 kDa, functioned as an effective lubricant at a cartilage-cartilage interface, but only under static conditions where the intrinsic biphasic lubrication was depleted. Despite the absolute values of μ_{static} for both HA and PBS being ~3X less than those reported here, which may be attributable to differences in the test configuration and protocols, these results support

those of the present study, that HA contributes to boundary lubrication. Similarly, Jay *et al.* [28] demonstrated Healon, an uncrosslinked form of HA, lowered μ from ~ 0.14 in PBS to ~ 0.07 at 3340 μg/ml, but not to the level of μ ~ 0.02 in SF, at a latex-glass interface under boundary lubricating conditions. This trend is similar to that observed in the present study, although again, the absolute value of μ is dissimilar to the $<\mu_{kinetic,\ Neq}>$ ~ 0.12 in 3330 μg/ml HA reported here (Fig. 6.3B). Such differences may be due to potentially specific interactions of HA with native articular cartilage surfaces, as postulated for PRG4 above. Certain synthetic surfaces may not be appropriate for testing the boundary lubricating ability of HA, since HA cannot contribute to boundary lubrication if it cannot first bind to the test surfaces [68].

Several other studies have reported HA to be both effective [20, 41], and ineffective [36, 39, 52, 66] as a boundary lubricant using different whole joint test apparatuses where several modes of lubrication were likely operative. The conflicting results of these studies support the disposition that characterization of a test configuration, surfaces, and mode of lubrication is important prior to making quantitative, mechanistic statements about putative boundary lubricants of articular cartilage. Accordingly, the test configuration and protocol used in the present study was characterized previously to demonstrate a boundary mode of lubrication was dominant (Chapter 5). Therefore, HA indeed does contribute, in a dose-dependent manner, to the boundary lubrication of articular cartilage. Additional pilot studies indicated that HA adsorbed to the articular surface of samples was able to contribute to boundary lubrication even without HA in the test bath, since samples soaked in HA, then rinsed and tested in PBS, still had a low μ. These studies suggest that HA was

retained at or between the articular cartilage surfaces under relative motion during testing.

The results indicating SAPL, in the form of DPPC, does not significantly contribute to the boundary lubrication articular cartilage, either alone or in combination with other SF constituents tested here, provide insight into to the controversial role of SAPL as a physiological boundary lubricant of articular cartilage. DPPC at ~ 0.35 mg/ml has been shown to slightly lower friction at a cartilage-steel interface [49], and phosphatidyl choline at 10 mg/ml dramatically reduced μ to ~0.016 (compared to μ ~ 0.028 in bovine SF), at a latex-glass interface under boundary lubricating conditions [27]. In the present study, SAPL in the form of DPPC at the physiological concentration of 200 μg/ml did not significantly lower $<\mu_{kinetic,\ Neq}>$ alone (Fig. 6.5B), which was still ~5-fold greater than $<\mu_{kinetic,\ Neq}>$ in SF, or in combination with HA and PRG4 (Fig. 6.6B). Additionally, because the PRG4 preparation tested in the present study was free of SAPL in appreciable quantities, SAPL was not indirectly contributing to the boundary lubricating ability of PRG4, as postulated by others [63]. However, the boundary lubrication ability of additional forms of SAPL [56] at a cartilage-cartilage interface, in various combinations, still remains to be determined.

HA and PRG4 synergistically lowered friction at the cartilage-cartilage interface tested here, presumably due to molecular interactions facilitating a molecular distribution of shear at the articular surfaces. Adsorbed layers of HA at the articular surface may have facilitated sliding [5], due to their inherent slipperiness and ease of disentangling [6], and therefore reduced friction between asperities in contact. The O-

linked oligosaccharides in the central mucin-like domain of PRG4 have been shown to contribute to the protein's boundary lubricating ability [29], possibly due to repulsive hydration forces [7, 31] or charge repulsion [7]. Jay *et al.* [31] previously reported HA and PRG4 acted synergistically together, with HA enabling PRG4 to lubricate under higher contact pressures at a latex-glass interface under boundary lubricating conditions. In that study, PRG4 appeared to reduce the number of intermolecular interactions between HA, since the viscosity of HA was reduced by the addition of PRG4. Hydrophobic attraction of PRG4 molecules along the length of HA was suggested as a possible mechanism for this interaction, although PRG4 does contain other putative binding domains as well [33]. In the present study, these interactions appeared specific since the subsequence addition of control proteins (albumins and globulins) at a physiological concentration did not further lower μ (data not shown).

These results suggest that the repulsive force generated at the individual contact asperities, on the articular cartilage surfaces, coated in HA and PRG4 was greater than that at asperities coated in either HA or PRG4 alone. The maintenance of SF's lubricating function at a 3-fold decrease in constituent concentration suggests the articular surface was completely coated by the physiological concentrations of HA and/or PRG4 used here. Assuming the number and area of the contact asperities, completely covered in lubricant molecules, remained similar between tests, the assertion regarding the magnitude of the repulsive force generated with HA and PRG4 would follow. This repulsive force may have been provided by the structuring of water at the articular surface by the aggregation and/or interaction of PRG4 and HA [25]. This theory is supported by the visualization of a reversible fringe pattern, indicative

of liquid crystal formation, imaged at the surface of pressurized cartilage by confocal laser microscopy [37]. Regardless of how the repulsive force is generated, it may indirectly provide protection to chondrocytes from wear and mechanical disturbances *in vivo* by reducing surface tissue shear.

The results of this study collectively provide insight into the nature of the boundary lubrication of articular cartilage with SF and its constituents. The maintenance of SF's boundary lubricating ability, at the cartilage-cartilage interface tested here, with a 3-fold decrease in constituent concentration suggests the lubricant molecules in SF may be present in excess. However, the rapid decline in boundary lubricating ability with a further decrease in constituent concentration suggests that such an alteration, which can occur in injury and disease [1, 11, 13, 43, 51, 71], can impair lubricating function. The combination of SF constituents HA, PRG4, and SAPL at physiological concentrations approaching, but not fully replicating, the boundary lubricating ability of SF suggests additional necessary lubricant molecules and/or complexes remain to be identified. More than one specific molecule contributing to the boundary lubrication of articular cartilage is not particularly surprising given the variety of interactions that can occur between the many molecules present in SF and at the articular surface [18, 34, 40, 47, 50, 55, 70]. Future studies that assess wear in addition to friction, and the accompanying surface tissue shear, will be helpful in elucidating the functional lubrication mechanisms in articular joints, which are relevant to both the homeostatic maintenance of healthy joints as well as the pathogenic processes in arthritic disease.

6.6 Acknowledgments

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CHAPTER 7

CONCLUSIONS

7.1 Summary of Findings

The overall motivation of this dissertation work was to contribute to the understanding of PRG4 metabolism by chondrocytes of the superficial zone of cartilage, and the mechanics of, and contributors to, boundary lubrication of articular cartilage. The major findings were:

1. The phenotype of PRG4 secretion by chondrocytes is generally maintained in explant, monolayer, and transplant culture, in that PRG4 is expressed to a much greater degree by chondrocytes from the superficial zone than by those from the middle and deep zones.
    a. Ascorbic acid stimulated PRG4 secretion in superficial cells.
    b. PRG4 was immunolocalized to superficial cells.
2. PRG4 secretion by chondrocytes near the articular surface is highly inhibited by IL-1α and stimulated by TGF-β1, in a dose-dependent manner, in explant cultures of cartilage.
    a. PRG4 staining was only evident for superficial disks in regions at or near the articular surface, and was absent from the deeper regions of superficial disks and also absent from middle disks.

b. The number of chondrocytes staining positive for PRG4 generally was consistent with the observed levels of PRG4 appearing in the medium.

3. PRG4 expression in chondrocytes near the articular surface is also highly regulated by IL-1α and TGF-β1 (but not IGF-I) in explant cultures of cartilage from the superficial zone, and the amount of PRG4 bound at the articular surface is modulated by culture with FBS.

a. PRG4 expression in chondrocytes is strongly correlated with PRG4 secretion.

b. The amount of PRG4 bound at the articular surface does not correlate with PRG4 secretion.

4. A boundary lubrication mode is operational at a depressurized articular cartilage-on-cartilage interface with an effective velocity (v_{eff}) = 0.3 mm/s, and a compression level ($1-\Lambda_Z$) = 18% for the annular geometry used here, since $<\mu_{kinetic}>$ was relatively invariant with v_{eff} and $1-\Lambda_Z$, a defining feature of boundary lubrication.

a. $<\mu_{static,\ Neq}>$ increased with pre-sliding durations in PBS and SF.

b. SF functioned as an effective friction-lowering boundary lubricant for native articular cartilage surfaces, $<\mu_{kinetic,\ Neq}> \sim 0.02$.

5. SF constituents contribute, individually and in combination, both at physiological and pathophysiological concentrations, to the boundary lubrication of opposing articular cartilage surfaces.

a. SF maintained its boundary lubricating ability at a 3-fold decrease in constituent concentration.

b. Both HA and PRG4 contributed independently to a low μ in a dose-dependent manner.

c. The combination HA, PRG4, and SAPL at physiological concentrations approached, but did not fully replicate, the boundary lubricating ability of SF.

7.2 Discussion

Current treatments do not appear to change the course of OA, with the composition, structure, and mechanical properties of the articular cartilage remaining abnormal [4]. Although the study in Chapter 2 did not assess the necessary integration of transplanted cells with the cartilage substrate for actual resurfacing, the transplantation of a cell-laden patch with the appropriate cell phenotype may facilitate repair. Resurfacing damaged opposed surfaces with specialized cells secreting a lubricating molecule and thus restoring functional lubrication may be generally applicable to many tissues in the body that slide against an opposed surface. Additionally, the up-regulation of PRG4 synthesis by superficial chondrocytes with ascorbic acid may have implications for cartilage homeostasis and prevention of osteoarthritic disease. A high dietary intake of vitamin C has been suggested to slow the progression of OA in guinea pigs [18, 25] and humans [16]. Possible mechanisms include modulating oxidative stress, participating in immune responses, and contributing to cellular differentiation [27]. Taken together with the tribologic

function of PRG4 in the joint [8, 10, 21, 28], it is possible that vitamin C slows the progression of OA due to its up-regulation of PRG4 synthesis.

The results of Chapters 3 and 4 indicate that PRG4 synthesis and secretion by chondrocytes near the articular surface is highly regulated by IL-1α and TGF-β1 in explant cultures of cartilage, and represent a step toward understanding the regulated role of PRG4 in cartilage during normal growth, homeostasis, and pathology. This marked regulation may alter the homeostatic balance of PRG4 at the surface of articular cartilage and in joints. The ability to modulate the dynamic regulation of the lubricant PRG4, whether in homeostasis or degeneration, may ultimately be useful in prolonging the maintenance or slowing the deterioration of articular cartilage's critical mechanical functions at the end of long bones. Additionally, the combination of chemical and mechanical factors to stimulate PRG4 expression in chondrocytes near the articular surface may be useful for creating tissue engineered cartilage from isolated sub-populations [14] with a functional lubricating surface.

The use of fresh osteochondral fragments in the annulus-on-disk rotational test configuration in Chapter 5 required attention to several experimental and theoretical issues. For example, the cyclical nature of the axial load $|N|$ during rotation (after initial fluid depressurization following axial compression), and the effects of rotation on the torque $|\tau|$ appeared to be explained predominantly by fluid pressurization during rotation. Accordingly, $<\mu_{kinetic,\ Neq}>$ is an appropriate measure of the frictional response of articular cartilage, minimizing the effects of fluid pressurization, under boundary lubricating conditions, especially for tests at the lower effective sliding velocities. Therefore, with attention to test sample preparation, and subsequent

characterization of friction properties, fresh osteochondral samples from non-apposing locations within the synovial joint, can be tested *in vitro* to analyze boundary lubrication at articulating cartilage surfaces.

The results from Chapter 6 collectively provide insight into the nature of the boundary lubrication of articular cartilage with SF and its constituents. The maintenance of SF's boundary lubricating ability, at the cartilage-cartilage interface tested, with a 3-fold decrease in constituent concentration suggests the lubricant molecules in SF may be present in excess. However, the rapid decline in boundary lubricating ability with a further decrease in constituent concentration suggests that such an alteration, which can occur in injury and disease [1, 5, 6, 15, 20, 30], can impair lubricating function. The combination of SF constituents HA, PRG4, and SAPL at physiological concentrations approaching, but did not fully replicating, the boundary lubricating ability of SF suggests additional necessary lubricant molecules and/or complexes remain to be identified.

7.3 Future Work

The work presented in this dissertation can be expanded upon in a number of ways. A detailed biochemical characterization of the functionally relevant mucin characteristics, such as multimerization and glycosylation [7, 29], of the PRG4 molecules secreted by chondrocytes would expand upon the framework established in Chapter 2. A multimerization potential is present in PRG4 due to the presence of cysteine-rich domains at both N- and C-terminals, along with an unpaired cysteine at

the C-terminal [23]. Indeed, a preliminary characterization of the various forms of PRG4 within joint tissues and fluid has confirmed that native PRG4 is present in disulfide bonded multimeric complexes as well as different monomer glycoforms [19]. Biochemical characterization of PRG4 molecules and/or fragments [13], secreted by chondrocytes cultured with the physiological cytokines examined in Chapters 3 and 4, as well as others, would also further the understanding of their regulatory effects. Similarly, the characterization of PRG4 metabolism by other cell types within the synovial joint, such as synovial fibroblasts [9] and meniscal cells [24], cultured alone or in combination, with or without cytokine stimulation, would provide insight into the potential distinct contributions of PRG4 by these various cell types and their regulatory effects on each other.

With the boundary lubrication test developed in Chapter 5, and the above mentioned biochemical characterization of monomeric glycoforms and multimeric aggregates of PRG4, the structure-function relationship governing the boundary lubricating ability of PRG4 could then be delineated. Indeed, preliminary studies indicate that the boundary lubricating ability of PRG4 is structurally mediated, through disulfide bonds, potentially inter-molecular in nature facilitating multimeric aggregate formation (Appendix A) [22]. Additionally, the potentially specific interactions of these molecular variant forms of PRG4 with other lubricant molecules, such as HA (Chapter 6), and their effect on boundary lubrication could be determined as well. Collectively, these types of mechanistic studies examining the structure-function relationship of lubricant molecules would greatly enhance the understanding of the boundary lubricating mechanism of articular cartilage in homeostasis.

Moreover, these types of mechanistic studies could be extended to pathological SF with diminished lubricating function where the SF concentration and composition of PRG4 molecular variants and other lubricating molecules may be altered [6, 11, 12].

Additional studies are needed to determine if a normal, non-degenerate articular surface is necessary for functional boundary lubrication. The results in Chapter 6 demonstrate alterations in SF constituent concentration can alter boundary lubricating ability, yet it remains to be determined if the altered structure (fissures and fibrillation [17]), and composition (low GAG content [3]) characteristic of OA cartilage tissue affect boundary lubrication function. These types of studies in combination with those mentioned above (examining healthy cartilage and pathological SF) might provide insight into the age old question of whether loss of lubrication leads to joint degeneration, or if early joint degeneration lead to changes in the frictional properties of articular cartilage.

The development and characterization of larger scale, more complex friction tests at the joint level might be useful for testing lubricants under different modes of lubrication at a cartilage-cartilage interface as well [2]. Such tests, after careful characterization, would provide the necessary tools to identify potentially mode-specific lubrication function of various SF molecules. Additionally, characterization of the surface tissue shear strain during articulation would provide insight into the potential protection of chondrocytes from wear and mechanical disturbances *in vivo*.

Finally, while friction is generally simpler to measure, wear is the ultimate metric of cartilage degeneration. Although SF does appear to confer low wear properties to articular cartilage [26], in addition to low friction properties, low friction

does not necessarily imply low wear. Therefore, additional studies that assess the wear of articular cartilage in various test lubricants are needed. These studies while be paramount in delineating potentially specific lubrication functions (i.e. low friction and/or low wear) of various molecules. Understanding the governing mechanisms of both friction and wear of articular cartilage is relevant to both the homeostatic maintenance of healthy joints as well as the pathogenic processes in arthritic disease.

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APPENDIX A

PROTEOGLYCAN 4 BOUNDARY LUBRICATION OF ARTICULAR CARTILAGE: ROLE OF DISULFIDE BONDING AND CHONDROITIN SULFATE

A.1 Abstract

Objective. Determine if the friction lowering effect of PRG4, in a boundary mode of lubrication, depends on disulfide bonding and potential substitution with chondroitin sulfate.

Methods. PRG4 was prepared from superficial cartilage disks harvested from immature bovine knee joints. Portions of PRG4 were reduced and alkylated, and termed PRG4+R/A. Other portions of PRG4 were treated with chondroitinase ABC, and termed PRG4+C-ABC. Lubricants were tested in a cartilage-on-cartilage friction test in the boundary lubrication regime using fresh adult bovine tissue. Static, $\mu_{static,\ Neq}$, and kinetic, $<\mu_{kinetic,\ Neq}>$, friction coefficients were calculated.

Results. $\mu_{static,\ Neq}$ increased with increasing pre-sliding duration, whereas $<\mu_{kinetic,\ Neq}>$ was steady. Values of $<\mu_{kinetic,\ Neq}>$ decreased significantly from 0.25±0.01 in PBS to 0.13±0.01 in PRG4+R/A, to similar values of 0.098±0.004 in PRG4+CABC and 0.086±0.005 in PRG4, down to 0.022±0.001 in SF.

Conclusion. Reduction and alkylation can alter the boundary lubrication properties of a characterized PRG4 preparation at opposed articular cartilage surfaces. These results suggest that disulfide bonds and/or tertiary protein structure modulate the lubrication function of PRG4 multimers and/or monomers.

A.2 Introduction

PRG4 proteins are composed of multiple domains, including the large central mucin like one [4], that may contribute to its boundary lubricating properties at an articular cartilage-cartilage interface. These include peripheral cysteine rich domains, as well as a central chondroitin sulfate (CS) substitution site [1, 3, 5].

The disulfide residues of PRG4 may contribute to the lubrication function of PRG4 by facilitating disulfide bonding, either intra-molecularly, or inter-molecularly forming multimeric complexes. The functional biological properties of mucins are generally determined by specialized glycosylation patterns, and the extent of multimeric complex formation through intermolecular disulfide bonds [2]. This multimerization potential is present in PRG4 due to the cysteine-rich domains at both N- and C-terminals, along with an unpaired cysteine at the C-terminal [8]. Indeed, PRG4 is secreted in various monomeric glycoforms and multimeric aggregates [6]. One approach to assessing the functional role of formed disulfide bridges is to reduce and alkylate these molecular structures, (which would reduce multimers to monomers and/or cause carboxymethylation and prevent binding of a free cysteine group), prior to lubrication testing.

The CS substation site of PRG4, at residue Ser244 (human sequence) within the motif DEAGSG, may mediate its lubrication properties as well by promoting attachment to extracellular matrix [1] components at the articular surface, such as collagen [9] and hyaluronan [7]. One approach to assess the functional role of CS molecules is to remove them with chondroitinase ABC (an enzyme that catalyzes the

conversion of CS, dermatin sulfate, and hyaluronate glycosaminoglycans to Δ4,5 unsaturated disaccharides by an elimination reaction [11]), prior to lubrication testing.

The objectives of this study were therefore to determine if the friction lowering effect of PRG4, in a boundary mode of lubrication, depends on disulfide bonding and potential substitution with CS.

A.3 Materials and Methods

Materials. Materials for tissue harvest and culture, and lubrication testing were obtained as described previously (Chapter 5, 6).

Lubricant Preparation & Characterization. PRG4 was prepared from superficial cartilage disks harvested from immature bovine knee joints (Chapter 6).

Portions of PRG4 were reduced and alkylated, and termed PRG4+R/A. Samples were first suspended in 4M guanidine, 100mM Tris, 1mM EDTA, pH 8.5, then reduced with 10 mM dithiothreitol for 2 hr at 60°C, and alkylated with 40 mM iodoacetimide (from a 10X stock in distilled water) for 2 hr in a dark room. Samples were then buffer exchanged into PBS using a 10 kDa MW cutoff filter, and diluted to a concentration of 450 μg/ml with PBS.

Other portions of PRG4 were treated with chondroitinase ABC, and termed PRG4+C-ABC. Samples were depleted of CS by incubation with 0.02 U/ml of protease-free chondroitinase-ABC (Seikagaku America, East Falmouth, MA) in 0.05M Tris, 0.01M NaAc, 0.002M EDTA, 0.2 mg/ml BSA, pH 7.4 for 60 min at 37°C. Samples were then treated with 0.001M $ZnCl_2$ on ice to deactivate C-ABC, and diluted to a concentration of 450 μg/ml with PBS.

Sample Preparation. Fresh osteochondral samples (n=8) were prepared for friction testing from the patellofemoral groove of 2 skeletally bovine stifle joints, and bathed in test lubricant, as described previously (Chapter 6).

Lubrication Test. Samples were placed in apposition, preconditioned, compressed, allowed to stress relax, and tested by rotating ±2 revolutions with various pre-sliding durations, as described previously (Chapter 6, Fig. 6.1). Lubrication tests were preformed in PBS, 450 μg/ml PRG4+R/A, 450 μg/ml PRG4+C-ABC, 450 μg/ml PRG4, and then SF.

Data Analysis. Static, $\mu_{static,\ Neq}$, and kinetic, $<\mu_{kinetic,\ Neq}>$, friction coefficients, and normal stress (σ) were calculated as described previously (Chapter 5).

Data are presented as mean±SEM. The effects of test lubricant, and T_{ps} (as a repeated factor), on the friction coefficients were assessed by ANOVA. As described previously, in all test lubricants, $<\mu_{kinetic,\ Neq}>$ varied slightly with T_{ps} ($p<0.001$), with values at T_{ps} = 1.2 s being similar to those at T_{ps} = 1200 s (on average within 10±5%, or 0.012±0.010 of values ranging from ~0.02 – 0.28, mean±SD). Therefore, for brevity and clarity, $<\mu_{kinetic,\ Neq}>$ data is presented at T_{ps} = 1.2 s only. Accordingly, the effect of test lubricant on $<\mu_{kinetic,\ Neq}>$ at T_{ps} at = 1.2 s was assessed by ANOVA, with Tukey post-hoc testing. Statistical analysis was implemented with Systat 10.2 (Systat Software, Inc., Richmond, CA).

A.4 Results

Effect of Chemical Treatment. Friction coefficients were modulated by chemical treatment of PRG4 (Fig. A.1).

$<\mu_{static,\ Neq}>$ varied with test lubricant ($p<0.001$) and T_{ps} ($p<0.001$), with an interaction effect ($p<0.001$) (Fig. A.1A). As described previously (Chapter 6), $\mu_{static,\ Neq}$ increased with increasing T_{ps}, and appeared to approach $<\mu_{kinetic,\ Neq}>$ asymptotically as $T_{ps} \rightarrow 0$. Values of $\mu_{static,\ Neq}$ were greatest in PBS, ranging from 0.36±0.02 to 0.58±0.02 with increasing T_{ps}, and least in SF, ranging from 0.038±0.003 to 0.29±0.02. Values of $\mu_{static,\ Neq}$ were similar in PRG4+R/A, PRG4+C-ABC and PRG4 at T_{ps}= 1200 s, ~ 0.40, and tended to diverge at T_{ps}= 1.2 s, appearing less in PRG4+C-ABC and PRG4, ~0.13, compared to that in PRG4+R-ABC, ~0.20.

$<\mu_{kinetic,\ Neq}>$ at T_{ps} = 1.2 s varied with test lubricant ($p<0.001$) (Fig. A.1B). Values of $<\mu_{kinetic,\ Neq}>$ decreased significantly from 0.25±0.01 in PBS to 0.13±0.01 in PRG4+R/A ($p<0.001$), to similar values of 0.098±0.004 in PRG4+CABC ($p<0.05$) and 0.086±0.005 in PRG4 ($p<0.01$), down to 0.022±0.001 in SF ($p<0.001$). The values of $<\mu_{kinetic,\ Neq}>$ in PRG4+C-ABC and PRG4 were not significantly different from each other ($p=0.74$).

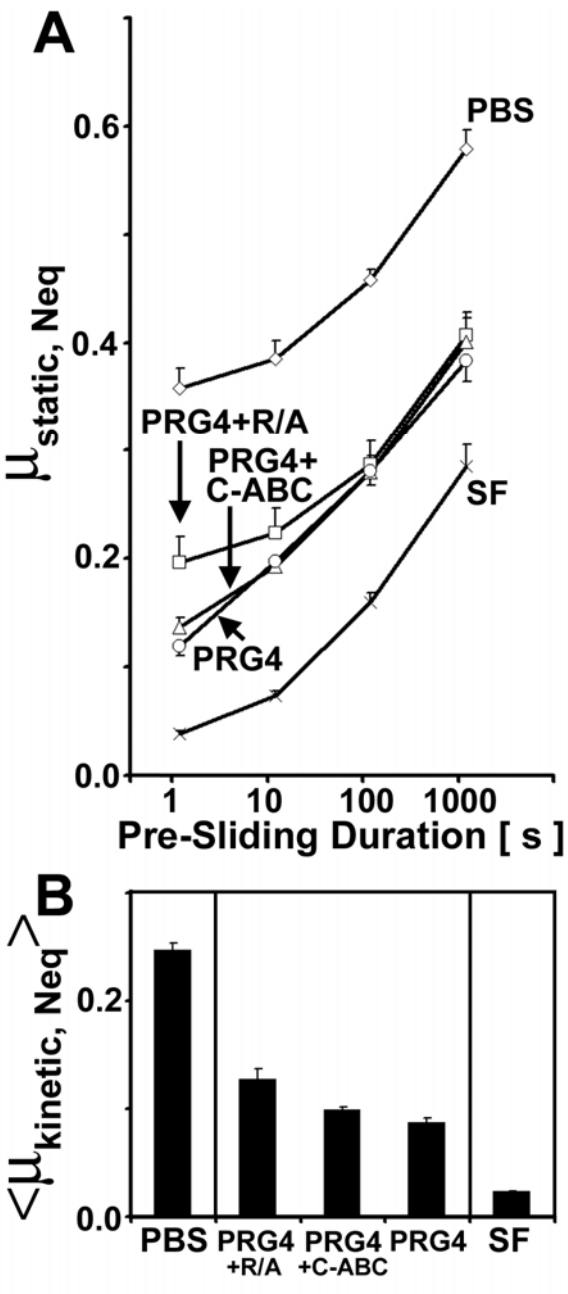

Figure A.1: Effect of reduction and alkylation (R/A) and chondroitinase ABC (C-ABC) on the articular cartilage boundary lubricating ability of PRG4. Static, $\mu_{static, Neq}$ (**A**) and kinetic, $<\mu_{kinetic, Neq}>$ at a pre-sliding duration, T_{ps}, of 1.2 s (**B**) friction coefficients in PBS, 450 μg/ml PRG4+R/A, 450 μg/ml PRG4+C-ABC, 450 μg/ml PRG4, and SF. Mean ± SEM, n=8.

A.5 Discussion

The results described here indicate that reduction and alkylation can alter the boundary lubrication properties of a characterized PRG4 preparation at opposed articular cartilage surfaces. The boundary lubricating ability of PRG4 at 450 μg/ml was confirmed. Values of $\mu_{static,\ Neq}$ increased with T_{ps}, values of $<\mu_{kinetic,\ Neq}>$ were steady with T_{ps}, and both were greatest in PBS and lowest in SF. The values of $\mu_{static,\ Neq}$ = 0.12 and $<\mu_{kinetic,\ Neq}>$ = 0.086, at T_{ps} = 1.2 s, in 450 μg/ml PRG4 increased significantly, by ~ 50%, to 0.20 and 0.13 in PRG4+R/A, respectively. Conversely, chondroitinase ABC treatment of PRG4 (PRG4+C-ABC) did not significantly increase either $\mu_{static,\ Neq}$ or $<\mu_{kinetic,\ Neq}>$. These results suggest that disulfide bonds, either intra-molecular, or inter-molecular, modulate the boundary lubricating ability of PRG4.

The interpretation of these results may be limited by the composition of PRG4 monomers and multimers within the purified preparation used for lubrication testing. As described previously (Chapter 6), the major immunoreactive band in the purified PRG4 preparation was a monomeric ~345 kDa form. However, PRG4 multimers have been visualized, in bovine SF and extract from articular cartilage, using agarose gel electrophoresis [6], which is more appropriate for the separation and visualization of PRG4 multimers. Therefore, the apparent absence of immunoreactive PRG4 multimers on SDS-PAGE, as observed in the present study, does not preclude the presence of PRG4 multimers in the purified preparation used for lubrication testing. Accordingly, the potentially specific, functional roles of the PRG4 multimers and

monomers in boundary lubrication, remain to be determined. Nevertheless, the decrease in PRG4's boundary lubrication function after reduction and alkylation, as assessed by an increase in $<\mu_{kinetic,\ Neq}>$, indicates disulfide bonds and/or tertiary protein structure modulate the lubrication function of PRG4 multimers and/or monomers.

The results described here are consistent with and extend several previous studies. In PRG4, $\mu_{static,\ Neq}$ values increasing from 0.12 to 0.28 with increasing T_{ps}, and $<\mu_{kinetic,\ Neq}> = 0.086$ and at $T_{ps} = 1.2$ s, are similar to those obtained previously (Chapter 6), $\mu_{static,\ Neq} = 0.16\pm0.04$ to 0.41 ± 0.02 and $<\mu_{kinetic,\ Neq}> = 0.10\pm0.02$, respectively. Equilibrium σ values obtained here, 0.13±0.02 MPa, are similar to those obtained previously as well, 0.11±0.04 MPa (mean±SD), suggesting incubation of the samples in chemically treated lubricants did not alter their mechanical properties. Swann *et al.* previously demonstrated reduction and alkylation of PRG4, isolated from SF, resulted in decreased lubricating ability (assessed at a cartilage-glass interface) and adsorption to the articular surface [10]. Swann suggested this inhibition of adsorption to the articular surface was at least partly responsible for the decreased lubricating ability of PRG4 after reduction and alkylation. These results are consistent with those of the present study, where $<\mu_{kinetic,\ Neq}>$ increased by ~50% with reduction and alkylation, and provide insight into why this effect was absent on $\mu_{static,\ Neq}$ at long T_{ps}. Reduction and alkylation may have inhibited PRG4 adsorption to the articular surfaces tested here as well, resulting in increased friction coefficients at short T_{ps}. At longer T_{ps}, potential non-specific deposition of unfolded or monomeric PRG4 to the

articular surface, may have facilitated a decrease in the resistance to the start up of motion, as indicated by similar values of $\mu_{static, Neq}$ in untreated PRG4.

The results of this study suggest the dose-dependent boundary lubricating ability of PRG4 is structurally mediated. The disulfide bonded PRG4 multimers present in SF, composed of monomer glycovariants, from different PRG4 sources, may be functionally determinant structures [6]. However, the mechanistic role of intra-molecular vs. inter-molecular disulfide bonds play in mediating boundary lubrication, potentially through adsorption to the articular surface, remains to be determined. Elucidating the structure-function relationship of PRG4 at a molecular level is essential to further the understanding of the boundary lubricating mechanism of articular cartilage.

A.6 Acknowledgments

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APPENDIX B

EFFECT OF PRE-SLIDING DURATION ON KINETIC FRICTION COEFFICIENTS

B.1 Introduction

Kinetic friction coefficients, $<\mu_{kinetic,\ Neq}>$, were measured at pre-sliding durations, T_{ps}, ranging from 1200 – 1.2 s in various test lubricants studied in Chapter 6 and Appendix A. However, for brevity and clarity, only the data from T_{ps} = 1.2 s was shown. This data is now presented, as supplemental information.

B.2 Materials and Methods

Test lubricants including SF, HA, PRG4, and SAPL are described in Chapter 6. The 30 mg/ml of albumins and globulins (A&G) test lubricant was composed of 20 mg/ml bovine albumin (Cohn fraction V, essentially globulin free), 8 mg/ml globulins (Cohn fraction IV-1, predominantly α-globulin), and 2 mg/ml γ-globulin (Cohn Fraction II, III), all obtained from Sigma (St. Louis, MO). The test lubricants PRG4+R/A and PRG4+C-ABC are described in Appendix A. Data was obtained and analyzed as described in Chapter 6. Data are presented as mean±SEM.

B.3 Results

In all test lubricants, $<\mu_{kinetic,\ Neq}>$ varied with T_{ps} ($p<0.001$). As described in Chapter 6 and Appendix A, values of $<\mu_{kinetic,\ Neq}>$ at T_{ps} = 1.2 s were similar to those at T_{ps} = 1200 s (on average within ~10±11%, or 0.010±0.014 of values ranging from ~0.02 – 0.3, mean±SD) in SF (Fig. B.1), HA (Fig. B.2), PRG4 (Fig. B.3), SAPL (Fig. B.4), HA+PRG4+SAPL (Fig. B.5), HA+PRG4+SAPL+A&G (Fig. B.6), and PRG4+R/A or C-ABC (Fig. B.7). PBS+A&G was the lone test lubricant where $<\mu_{kinetic,\ Neq}>$ varied substantially with T_{ps} (Fig. B.6), appearing to increase with decreasing T_{ps}.

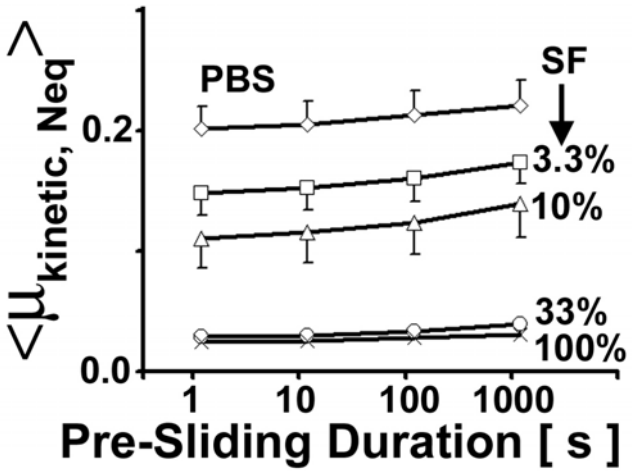

Figure B.1: Effect of pre-sliding duration, T_{ps}, on kinetic friction coefficients $<\mu_{kinetic, Neq}>$ in PBS and 3.3 - 100% SF. T_{ps} = 1.2 - 1200 s. Mean ± SEM, n=4-8.

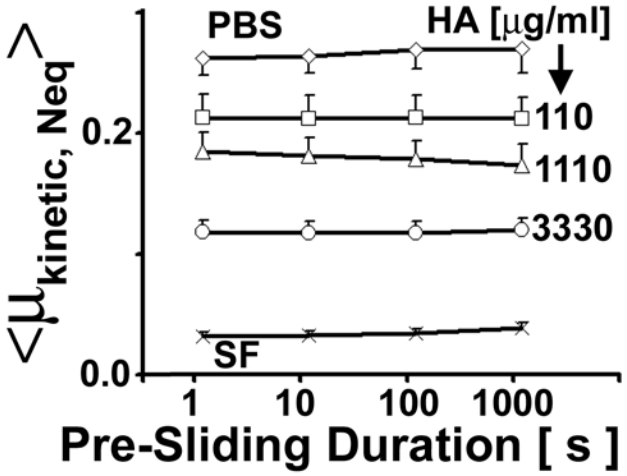

Figure B.2: Effect of pre-sliding duration, T_{ps}, on kinetic friction coefficients $<\mu_{kinetic, Neq}>$ in PBS, 110 – 3330 μg/ml HA, and SF. T_{ps} = 1.2 - 1200 s. Mean ± SEM, n=4-8.

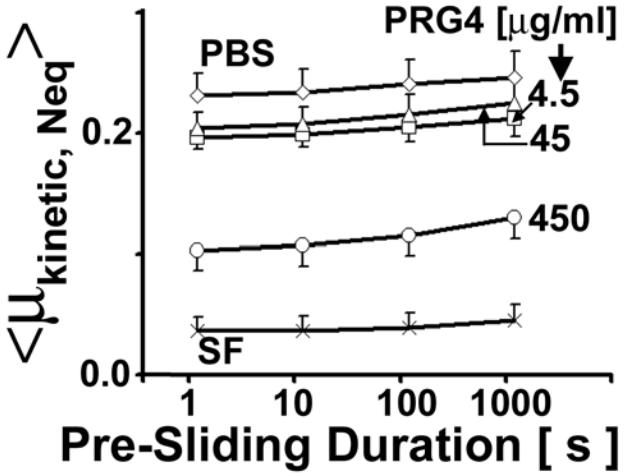

Figure B.3: Effect of pre-sliding duration, T_{ps}, on kinetic friction coefficients $<\mu_{kinetic, Neq}>$ in PBS, 4.5 - 450 μg/ml PRG4, and SF. T_{ps} = 1.2 - 1200 s. Mean ± SEM, n=8.

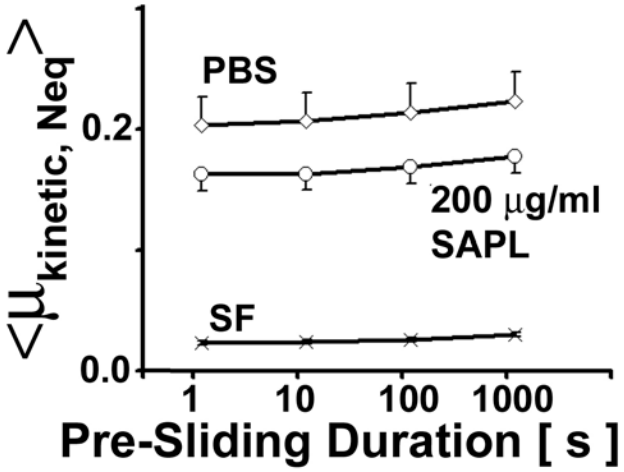

Figure B.4: Effect of pre-sliding duration, T_{ps}, on kinetic friction coefficients $<\mu_{kinetic, Neq}>$ in PBS, 200 μg/ml SAPL, and SF. T_{ps} = 1.2 - 1200 s. Mean ± SEM, n=8.

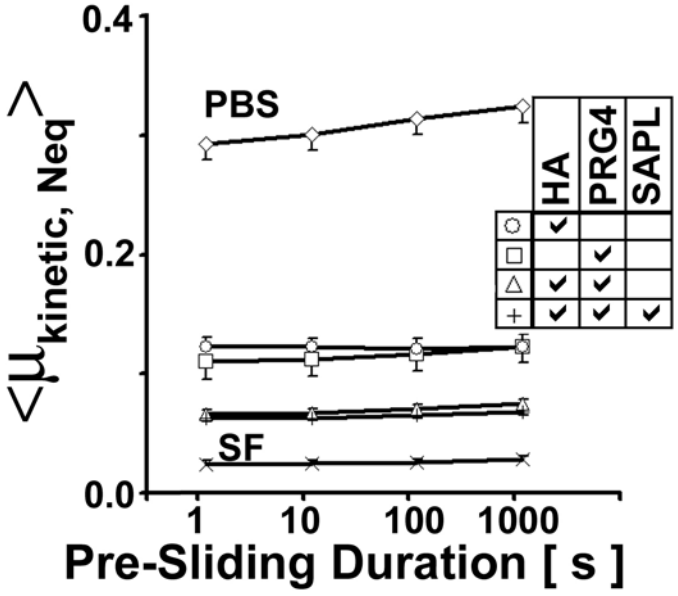

Figure B.5: Effect of pre-sliding duration, T_{ps}, on kinetic friction coefficients $<\mu_{kinetic, Neq}>$ in PBS, PBS + 3330 μg/ml HA, PBS + 450 μg/ml PRG4, PBS + HA + PRG4, PBS + HA + PRG4 + 200 μg/ml SAPL, and SF. T_{ps} = 1.2 - 1200 s. Mean ± SEM, n=4-8.

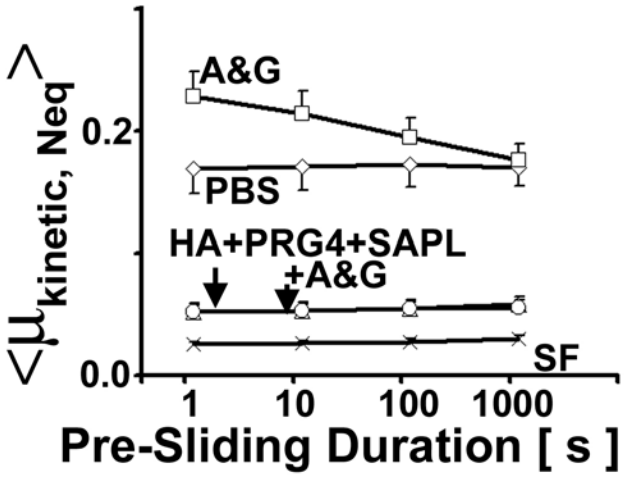

Figure B.6: Effect of pre-sliding duration, T_{ps}, on kinetic friction coefficients $<\mu_{kinetic, Neq}>$ in PBS, PBS + 30 mg/ml A&G, PBS + 3330 μg/ml HA + 450 μg/ml PRG4 + 200 μg/ml SAPL, PBS+HA+PRG4+SAPL+A&G, and SF. T_{ps} = 1.2 - 1200 s. Mean ± SEM, n=4-8.

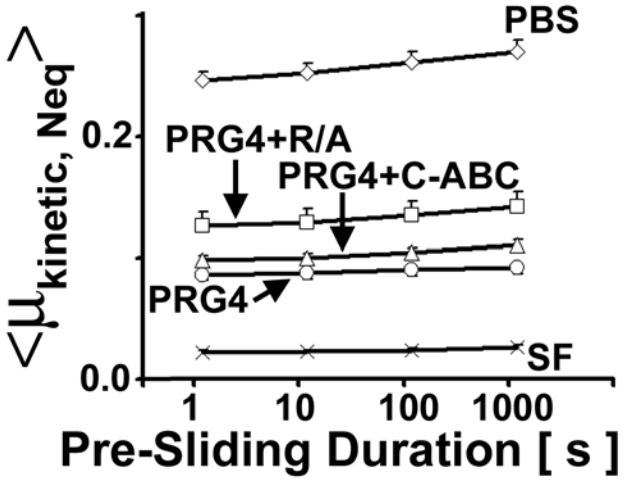

Figure B.7: Effect of pre-sliding duration, T_{ps}, on kinetic friction coefficients $<\mu_{kinetic, Neq}>$ in PBS, 450 μg/ml PRG4+R/A, 450 μg/ml PRG4+C-ABC, 450 μg/ml PRG4, and SF. T_{ps} = 1.2 - 1200 s. Mean ± SEM, n=8.

B.4 Discussion

The effects of the test lubricants, at all T_{ps}, are identical to those described in Chapter 6 and Appendix A for T_{ps} = 1.2 s only, except for PBS+A&G. The mechanism by which A&G, at physiological concentrations, increases friction at a cartilage-cartilage interface under boundary lubricating conditions at shorter T_{ps} remains to be determined.