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Lubricin as A Therapeutic and Potential Biomarker in Sepsis

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1. Introduction

a. Lubricin – original discoveries

Lubricin (also known as proteoglycan 4 or PRG4), expressed by the PRG4 gene, is a mucin-like 224 kDa glycoprotein known for its lubricating properties and was originally found within the synovial fluid of diarthrodial joints in bovine and human samples and secreted by synovial fibroblasts^{1–7}. Lubricin contains many O-linked glycosylations located in a central post-translationally modified mucin domain. The length of the mucin-like repeats in its structure varies across species but is generally proportional to the size of the animal^{4,6}. However, lubricin can also be expressed with several different isoforms in the N-terminus and each isoform may have different functions⁸. Like other mucins and mucin-like substances, lubricin prevents the attachment of cells to surfaces which enables its activity as a boundary lubricant by reducing friction⁹.

b. Friction and apoptosis

The ability of lubricin to exert its lubricating effects and decrease friction is dependent upon its concentration, indicated by an increase in joint friction with decreased lubricin concentrations⁴. Optimal concentrations of lubricin were found to be between 200–260 μ g/mL for proper lubricating activity and reduced friction coefficients in non-cartilage surfaces⁷.

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Patients born with a rare genetic mutation in the PRG4 gene display camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome which is characterized by increased joint friction, apoptosis and cellular morphological changes that eventually lead to joint failure^{10–12}. Lubricin null mice (*Prg4*^{−/−}) (the orthologous genetic model of CACP syndrome in humans) were bred in order to study joint mechanics and to examine histological abnormalities. *Prg4* gene trap (^{GT/GT}) mice have increased friction and activated caspase 3 staining in their chondrocytes, which is reversed after the *Prg4* gene is turned back on through Cre-mediated recombination¹³. The coefficient of friction and amount of activated caspase-3 staining in lubricin null mice given purified human synoviocyte lubricin was significantly lower than mice that were lubricin null littermates receiving only sham injections¹⁴. Moreover, lubricin null mice had higher levels of ONOO[−] in femoral head cartilage and synoviocytes when compared to controls¹⁴. Additionally, there was increased gene expression of the cytokines IL-1 β and TNF- α , and caspases 3, 6 and 7 in the cartilage of lubricin null mice compared to controls indicating the anti-inflammatory role of PRG4 in preventing apoptosis that is activated through the intrinsic pathway¹⁴.

Other studies reveal that lubricin has a cellular protective effect, as knee joints from lubricin null mice had increased numbers of apoptotic cells which was measured via anti-caspase-3 and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)¹⁵. In rats with anterior cruciate ligament transection (ACLT), expression levels of lubricin and deposition of lubricin on cartilage surfaces were significantly decreased when assessed at 3 and 5 weeks post transection¹⁶. At 3 weeks, exercised rats with ACLT had expression levels of lubricin that were even lower than rats that had undergone ACLT only, indicating increased damage in the joint¹⁶. Moreover, the rats with ACLT + exercise also showed an increase in activated caspase-3 staining of chondrocytes in comparison to ACLT only rats which was further increased at 5 weeks¹⁶. Interestingly, treatment of ACLT rats with intra-articular purified human lubricin significantly lowered activated caspase-3 staining of chondrocytes compared to those with no lubricin¹⁶.

Osteoarthritis (OA) is a common age-related degenerative disease of the joints that consists of cartilage degeneration, inflammation, and bone remodeling, characterized by pain, joint stiffness, and swelling¹⁷. The causes of OA are still not well understood but are thought to stem from genetic and orthopedic abnormalities, gender, metabolic diseases, environmental factors such as sports injuries, and obesity¹⁷. There is still an unmet need for treatment of patients with osteoarthritis, but several reports point to lubricin not only as a factor in the progression of the disease process and but also as a potential effective therapy for its treatment^{11,18,19}.

OA in humans presents with downregulated PRG4 in lateral femoral condyle cartilage, which may be location and isoform dependent²⁰. In a sheep model of osteoarthritis, lubricin immunostaining was decreased in cartilage which also corresponded with downregulated mRNA levels of PRG4 in comparison to sham treated animals¹⁹. Moreover, PRG4 was shown to prevent osteoarthritis in a mouse model using intra-articular injected adenoviral vectors conjugated to antibodies designed to over express PRG4^{21,22}. It appears that one of the mechanisms of cellular protection is via PRG4 inhibition of cellular pathways that lead to catabolism of cartilage²¹.

Rheumatoid arthritis (RA) is a type of arthritis that has autoimmune and inflammatory components²³. The symptoms of RA are very similar to those of OA however, the causes and co-morbidities are different^{23,24}. Autoimmunity leads to joint destruction and swelling in addition to inflammation of the synovium^{23,24}. Interestingly, lubricin concentrations in synovial fluid from both OA and RA patients is significantly lower than that of controls²⁵. Moreover, synovial fluid from patients with RA failed to lubricate rabbit knee joints after induced injury, indicating that there is a loss of lubricin that occurs within the RA disease process similar to that of OA²⁶.

These findings indicate the very significant role that PRG4 has in mitigating joint friction and in preventing chondrocyte cell death in normal and disease states. These studies also suggest that PRG4 should be considered as an intra-articular therapeutic and potential biomarker in patients that sustain joint related injuries or in patients with CACP, OA, and RA in order to provide a protective effect on the joint²⁷.

2. More recent discoveries

Although lubricin was originally found in cartilage and synovial fluid, and secreted by the synovial fibroblasts, it has since been found in other tissues such as lung, heart, liver, eye, uterus, cervix, prostate, bladder, and bone, indicating that it has a multifunctional role that is not limited solely to joints and friction reduction^{5,6,28}. Therefore, due to lubricin's ubiquitous nature, there have been numerous reports of lubricin as a potential therapeutic in several non-tribologic disorders which are described below²⁹.

a. Extra articular non-tribologic functions of lubricin

Bowel and bladder permeability—Interstitial cystitis-bladder pain syndrome (IC-BPS) is a relatively common disorder characterized by painful and increased frequency of urination from disruption of epithelial cells in the bladder. Irritable bowel syndrome (IBS) is a common disorder of the large intestine involving pain, cramping, bloating, gas, diarrhea and/or constipation and has a high co-morbidity with IC-BPS, indicating cross-talk of the disorders³⁰. Currently, patients with either IBS or IC-BPS have very limited treatment options^{30,31}. In rats, treatment with protamine sulfate (PS) induced bladder and subsequently colon permeability which mimic IC-BPS and IBS in humans³². When rats that were exposed to PS and later treated with lubricin, there was a reduction of the PS induced permeability in both the bladder and the colon²⁹. Therefore, lubricin could have potential as a therapeutic for patients with IC-BPS or IBS.

Patients that undergo abdominal surgery have a high chance of developing intra-abdominal lesions, with up to 20% requiring treatment despite newer less invasive surgical techniques^{33,34}. There are currently no proven methods to completely prevent the formation of intra-abdominal lesions resulting in a clinical need for improved therapies³⁵. Intra-abdominal adhesions created in rats via both cecal abrasion and cecal enterotomy with only the cecal abrasions leading to significant lesion formation³⁵. In rats with cecal abrasions, rhPRG4 treatment applied directly after surgery prevented both inflammation and fibrosis of the lesioned areas, indicating a potential role for lubricin as a post-operative treatment in patients that undergo abdominal surgeries³⁵.

b. Clinical uses for PRG4

Dry eye (xerophthalmia) and dry mouth (xerostomia) diseases—Dry eye (xerophthalmia) is a condition in which there are not enough tears produced in order to lubricate the ocular surface leading to discomfort, light sensitivity, burning sensations, inflammation, and potential eye damage ^{36,37}. There are several other conditions which lead to dry eye including the use of contact lenses, dry eye disease, Sjogren's syndrome, Stevens-Johnson syndrome, vitamin deficiencies, RA, and refractive surgery ³⁷. The current treatments for dry eye include hyaluronic acid (HA), polyvinyl alcohol, povidone, hydroxypropyl guar, and cellulose derivatives and treatment depends upon the cause of dry eye ³⁶. Dry eye typically has an inflammatory component and if it is severe enough, may need anti-inflammatory or immunosuppressant treatments ³⁶.

Dry mouth (xerostomia) is the feeling of oral dryness and most often occurs in older adult women³⁸. The most common complaints with xerostomia are difficulty talking, chewing and swallowing in addition to altered taste ³⁸. Some of the most common causes of xerostomia include cancer treatment, various medications, and Sjogren syndrome ³⁸.

Sjogren syndrome (SS) is an autoimmune disease that mainly results in both dry eye and dry mouth but can affect other organ systems ³⁹. Women are more effected by SS than men and it most often occurs in later years ³⁹. However, the causes of SS are not well known as it seems there are many contributing factors ³⁹. Due to the lubricating and anti-inflammatory properties of PRG4, several studies have indicated PRG4 as a therapeutic for both dry eye and dry mouth. It has been documented that lubricin deficiency in the eye has a negative impact on corneal functioning ²⁸. In lubricin null mice, there was a significant increase in fluorescein staining and inflammation compared to wild type animals, signifying that substantial corneal damage occurs without lubricin present ²⁸.

HA is commonly used in patients with dry eye and it is commercially available. Recently, a clinical trial indicated that lubricin is an excellent lubricating factor in patients with moderate dry eye disease and is more effective than HA at treating dry eye ⁴⁰. Moreover, a deficiency of lubricin as seen in lubricin null mice, led to increased corneal fluorescein staining, illustrating the need for lubricin to reduce friction by acting as a natural lubricant in the space between the cornea and eyelid ²⁸. Recently, cathepsin S (CTSS) was found to be increased in the tears of patients with SS ⁴¹. Interestingly, CTSS was found to decrease ocular lubrication via degradation of PRG4 in human tears ⁴². Therefore, it seems that one of the mechanisms of dry eye in SS patients occurs via a reduction of PRG4 at the ocular surface ⁴².

These results discussed above suggest that PRG4 would be a better treatment for dry eye than current treatments such as HA. Commercialization efforts are currently under way for production of PRG4 intended for lubricating eye and mouth drops by Lubris, LLC and Novartis in the case of the dry eye indication.

c. Anti-inflammatory actions of lubricin

TLR4, CD44, NF- κ B, NLRP3—Recently, lubricin has been studied to determine if it serves as a ligand for various receptors that are involved in cellular inflammatory cascades.

Several studies indicate that lubricin binds to the integrin CD44 and some members of the toll-like receptor (TLR) family which suggests a role for lubricin in regulating inflammatory cascades ^{43,44}. Moreover, as detailed below, PRG4 reduces nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) nuclear translocation and nod-like receptor pyrin domain containing 3 (NLRP3) inflammasome activation leading to decreases in the production of proinflammatory cytokines.

CD44 is a glycoprotein transmembrane cell surface receptor that is well-known for binding to hyaluronic acid (HA) and internalizing it via receptor-mediated endocytosis and is highly involved in inflammatory cascades ^{45–48}. CD44 signaling seems to have function in both pro-inflammatory and anti-inflammatory pathways, as studies show contradictory reports about its function depending upon ligand binding, cell type, and type of infection. For example, in CD44 knockout mice, renal dysfunction and inflammation was delayed after lipopolysaccharide (LPS) treatment ⁴⁹. On the contrary, CD44 deficient mice with *Escherichia coli* infections had increased lung inflammation and pro-inflammatory cytokine release ^{50,51}. Therefore, ligand binding to CD44 may elicit both pro and anti-inflammatory cascades depending upon cell type and other factors.

Besides HA, PRG4 was found to be one of CD44's ligands. Using direct ELISA, recombinant human PRG4 (rhPRG4) was found to bind to recombinant CD44 receptors with a higher affinity than CD44's known ligand, HA and was concentration dependent ⁴⁴. However, HA blocked the binding of rhPRG4 to CD44 in a concentration dependent manner ⁴⁴. Interestingly, removal of the mucin domain's sialic acid and O-glycosylations of rhPRG4 resulted in an even greater affinity of rhPRG4 to CD44. In the same study, RA fibroblast-like synoviocytes (RA-FLS) proliferation was induced via IL-1 β and TNF- α which was suppressed with rhPRG4 ⁴⁴. IM7, an antibody directed against CD44, blocked the suppressive effects of rhPRG4, further verifying that CD44 is one of PRG4's receptors ⁴⁴.

The toll-like receptors are a large 11-member family of receptors that are associated with host defense mechanisms for innate immunity ^{52,53}. The TLR family of receptors is responsible for the detection of pathogen-associated molecular patterns (PAMPs) from bacteria, fungi, and viruses and are considered one family of the pathogen-recognition receptors (PRRs) ⁵². Upon recognition of said pathogens, a cascade of immune events occurs in the host including cytokine release and transcriptional factor activation ⁵². Therefore, antagonists of the TLRs have been utilized as a means to reverse immune cascades in order to treat various immune related disorders ^{54,55}.

Both rhPRG4 and native human PRG4 (nhPRG4) bind to Toll-like receptors 2 and 4 (TLR2 and TLR4) and binding is concentration dependent which was verified via ELISA and via a TLR2/TLR4 HEK-293 reporter cell line ⁴³. Moreover, nhPRG4 was effectively able to block TLR2 and TLR4 activation when the agonists Pam3CSK4 and LPS were used ⁴³. TLR2 and TLR4 activation can also be stimulated via human synovial fluid (SF) samples from patients with OA and RA which are effectively blocked with both rhPRG4 and nhPRG4 ⁴³. Interestingly, OA and RA SF have naturally depleted levels of PRG4 which coincides with increased TLR2 and TLR4 activation ⁴³. Therefore, PRG4 seems to be an effective

antagonist of both TLR2 and TLR4, thus blocking downstream inflammatory cellular responses.

Activation of inflammatory pathways including ligand binding of both the TLRs and CD44 stimulates NF- κ B translocation into the nucleus which triggers a cascade of inflammatory related events^{44,56}. NF- κ B is a family of transcription factors and are well-known for being activated during innate and acquired immune responses and subsequently stimulate the release of chemokines, cytokines, anti-apoptotic proteins, and stress-response proteins^{56,57}. Moreover, these same chemokines, cytokines and proteins can also trigger NF- κ B translocation in a reciprocal fashion⁵⁶. However, more recent findings indicate that the role of NF- κ B is tissue specific and is implicated in the production of both inflammatory and anti-inflammatory genes spurring characterization of the canonical and alternative NF- κ B signaling pathways⁵⁶. These results indicate that the function of NF- κ B is difficult to elucidate and that activation of NF- κ B stimulates release of both pro and anti-inflammatory factors which would increase or decrease apoptosis depending upon cellular dynamics.

Recent reports indicate that lubricin can prevent NF- κ B translocation. For example, treatment of RA-FLS with TNF α induces NF- κ B translocation into the nucleus which can be blocked with both rhPRG4 or MG132 (a known inhibitor of NF- κ B translocation)⁴⁴. Moreover, blockade of NF- κ B translocation from rhPRG4 was significantly reduced with the IM7 antibody⁴⁴. Similarly, in THP-1 macrophages treated with monosodium urate (MSU) crystals, NF- κ B translocation was significantly increased which was reversed by PRG4 treatment⁵⁸.

The NLRP3 inflammasome is another facilitator of inflammatory cascades within the cell^{58–60}. NLRP3 can be stimulated by a variety of factors within the cell such as NF- κ B translocation and when “primed”, initiates pro-caspase-1 activity, creating the inflammasome that promotes the cleavage of pro-interleukin 1 beta (IL-1 β) and pro-interleukin 18 (IL-18) into their mature forms to further drive the inflammatory response^{58–60}. PRG4 inhibited both the inflammasome and pro-caspase-1 activation in macrophages that were induced into an inflammatory state via MSU crystals, which are typically deposited in joints in patients with gout⁵⁸.

Interestingly, pro-inflammatory cytokines and growth factors also play a role in regulating the production and release of lubricin^{61,62}. The cytokines tumor necrosis factor alpha (TNF- α) and IL-1 decrease both PRG4 mRNA and protein expression from both articular chondrocytes and synoviocytes whereas the growth factor TGF- β increased PRG4 mRNA expression^{61,62}. PRG4 has also been shown to regulate the release of various cytokines and chemokines⁵⁸. For example, MSU treated THP-1 macrophages induced significant increases in the cytokines IL-1 β and TNF- α , and the chemokines IL8 and MCP-1 all of which were significantly reduced with treatment of rhPRG4⁵⁸.

Due to the above findings, a role for PRG4 as an anti-inflammatory biologic has emerged and indicates that a lubricin deficiency may serve as a biomarker of inflammatory processes and inflammation. Lubricin’s antagonism with receptors of the TLR family and interaction with the CD44 receptor indicate that it may be an important treatment option in a variety of

disorders with an immunological basis (Figure 1). Therefore, further studies of rhPRG4 and its interaction in immune pathways have more recently been discovered as will be discussed in the next section.

d. Potential therapeutic role of PRG4 in sepsis

Sepsis background—Sepsis is a major health concern due to its high mortality rate and ineffective current treatments. Sepsis was previously technically defined as a syndrome resulting from systemic inflammatory response syndrome (SIRS) which stems from bacterial and viral infections creating a cascade of inflammatory responses within the host also known as the “cytokine storm”. However, newer reports have proposed that certain terminology be removed in order for clinicians to more accurately diagnosis sepsis and initiate treatment ⁶³. There are also cases in which sepsis can occur from non-infectious conditions such as pancreatitis, burns, and tissue injury ⁶⁴. In the absence of no adequate intervention or treatment, the inflammatory cascades and cytokine storm usually lead to septic shock, organ failure and death ⁶⁴.

Because sepsis is a highly multi-factorial syndrome, it is very difficult to treat and most treatments that have been tested in clinical trials were ineffective ⁶⁴. Another reason that sepsis is challenging to treat is the delay between the time the patient presents with symptoms to the time that white blood cell, blood cultures, and serum lactate test results are received, resulting in a delay of diagnosis ⁶⁵. Currently, results can take up to 72 hours and the tests are unreliable for sepsis diagnostics ^{65,66}. Presently, broad-spectrum antibiotics, vasopressors, and drugs to treat hypotension are administered to patients with suspected sepsis. Patients treated more quickly are then likely to have a more positive outcome ^{64,67}. Even with current sepsis treatments, there is still a 30% death rate of patients with sepsis, indicating the need for more effective treatments ⁶⁸.

Therefore, new biomarkers and detection techniques for earlier identification of sepsis are needed ^{69–72}. A new report details an antibody based small microelectrode biosensor to test levels of the cytokine Interleukin-6 (IL-6) within as little as 2.5 minutes. A significant improvement in treatment time for septic patients ⁷⁰. The biosensor can also be engineered to test multiple biomarkers at the same time which would significantly improve patient wait times and treatment paradigms ⁷⁰. Other types of biosensors have also been explored, including electrochemical, optical, and microfluidic based sensors ⁷³. However, the issues with biosensors include high cost, low sensitivity, and short shelf-life all of which create a hinderance on use in the clinic ⁷³. However, once the logistical issues are removed, biosensors will become a critical piece of sepsis diagnosis in the future.

e. Cytokine role in sepsis

role of IL-6 in sepsis—The glycoprotein, Interleukin-6 (IL-6) is a cytokine that is released during the body’s inflammatory response resulting from pathogen invasion or tissue damage ^{74,75}. IL-6 is secreted from many different cell types including leukocytes, vascular endothelial (VE) cells, mesenchymal cells, fibroblasts, osteoblasts and tumor cells ⁷⁶. IL-6 is produced and released via stimulation by other cytokines such as IL-1 β and TNF-alpha, via the host cell’s initial recognition of PAMPs through PRR’s, or from damage associated

molecular patterns (DAMPs) and acts as a warning signal to other cells ^{74,77}. IL-6 is typically thought of as a pro-inflammatory cytokine but does function in the anti-inflammatory pathway as well ⁷⁵. When IL-6 expression and secretion becomes dysregulated severe immune related disorders develop, one of them being sepsis ⁷⁷. Therefore, drugs, biologics and therapeutics that are able to block secretion and/or binding of IL-6 to its receptors have become a major research focus ⁷⁷.

Sepsis has two phases: the hyper-inflammatory and the immunosuppressive phases both of which contain different cytokine spikes⁷⁸. There are several cytokines that can be tested as biomarkers of the severity of a sepsis infection although IL-6 is considered one of the best early biomarkers for prognosis of both adult and neonate patients with a severe sepsis infection and/or septic shock ^{72,79–83}.

Therefore, based upon the aforementioned information, IL-6 stood out as an important marker for early cellular inflammatory responses to which lubricin could make a potential therapeutic and impact if it was able to reduce IL-6 protein and gene expression.

Results from our lab—Due to lubricin's role in the inflammatory pathways involving CD44, TLR4 and NLRP3 as discussed above, our lab has identified lubricin as a possible therapeutic for patients with sepsis due to its ability to lower both protein levels and gene expression of IL-6 in human umbilical vascular endothelial cells (HUVECs) and in human lung microvascular endothelial cells (HLMVECs) ⁸⁴. HUVECs and HLMVECs were treated with LPS to induce a high inflammatory state, resulting in significantly increased IL-6 protein levels and gene expression, both of which were reversed to control levels with 50, 100 and 150 µg/mL rhPRG4 treatment ⁸⁴. Moreover, HLMVECs were treated with plasma from septic patients to induce an inflammatory response and rhPRG4 significantly lowered IL-6 protein levels in almost 75% of the patients ⁸⁴. Because lubricin biosynthesis is downregulated by various cytokines including IL-1β and TNF-alpha, it is possible lubricin could not only serve as a therapeutic but also as a biomarker of inflammation and infections, including sepsis ⁶¹.

Conclusions

Lubricin's original role in the reduction of joint friction has evolved to a multitude of reports on the non-tribologic functions of lubricin, notably within inflammatory pathways. Lubricin is an antagonist of TLR2 and TLR4, which is one mechanism of its role in reducing inflammation. Moreover, lubricin also interacts with CD44 in order to block NF-κB translocation into the nucleus and formation of the NLRP3 inflammasome. Therefore, these results led to further research into lubricin as an anti-inflammatory and possible biomarker of sepsis. Lubricin effectively decreased protein levels and gene expression of IL-6, a prominent sepsis biomarker. We conclude that lubricin has the potential to be an effective therapeutic for many diseases with underlying inflammation, including but not limited to sepsis.

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Bullet Points:

- Lubricin is a lubricant and has a cellular protective effect as it prevents cellular death and decreases cellular inflammation
- Lubricin is an antagonist of TLR2 and TLR4 and gains entry into the cell via CD44 to block inflammatory processes indicating a therapeutic role
- Lubricin is downregulated by a number of cytokines, indicating it could serve as an inflammatory and sepsis biomarker

Synopsis

Proteoglycan 4 (aka PRG4 or Lubricin), a mucin-like glycoprotein, was originally classified as a lubricating substance within diarthrodial joints. More recently, lubricin has been found in other tissues and has been implicated in two inflammatory pathways within the cell, via the toll-like receptors (TLR) and CD44. Lubricin is an antagonist of TLR2 and TLR4 and gains entry into cells via the CD44 receptor. Because of lubricin's action on these receptors, downstream processes of inflammation are halted, thereby preventing release of cytokines (a hallmark of inflammation and sepsis) from the cell, indicating lubricin's role as a biomarker and possible therapeutic for sepsis.

Figure 1: Proposed mechanism of action of lubricin in inflammatory cascades.

Various ligands bind to both the TLRs and CD44, namely, lipopolysaccharide (LPS) and hyaluronic acid (HA). Activation of these receptors activate internal cellular pathways involved in inflammation. The inflammasome becomes activated and initiates conversion of pro IL-18 and pro-IL1β to their mature forms to be released from the cell. NF-κβ translocates to the nucleus which also initiates the production and release of other cytokines. When cells are treated with rhPRG4, these inflammatory cascades are halted. rhPRG4 strongly binds to both TLR2 and TLR4 as an antagonist and competitor with other ligands.

rhRPG4 also becomes internalized into the cell via CD44 allowing rhPRG4 to both prevent activation of the inflammasome and translocation of NF- κ B thereby preventing the formation of cytokines.

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