Effects of Stress Deprivation on Lubricin Synthesis and Gliding of Flexor Tendons in a Canine Model in Vivo

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Background: Lubricin facilitates boundary lubrication of cartilage. The synthesis of lubricin in cartilage is regulated by mechanical stimuli, especially shear force. Lubricin is also found in flexor tendons. However, little is known about the effect of mechanical loading on lubricin synthesis in tendons or about the function of lubricin in flexor tendons. The purpose of this study was to investigate the relationship of mechanical loading to lubricin expression and gliding resistance of flexor tendons.

Methods: Flexor tendons were harvested from canine forepaws that had been suspended without weight-bearing for twenty-one days and from the contralateral forepaws that had been allowed free motion. Lubricin expression in each flexor tendon was investigated with real-time RT-PCR (reverse transcription polymerase chain reaction) and immunohistochemistry. Lubricin in the flexor tendon was extracted and quantified with ELISA (enzyme-linked immunosorbent assay). The friction between the flexor tendon and the proximal pulley was measured.

Results: The non-weight-bearing flexor tendons had a 40% reduction of lubricin expression ($p < 0.01$) and content ($p < 0.01$) compared with the flexor tendons in the contralateral limb. However, the gliding resistance of the tendons in the non-weight-bearing limb was the same as that of the tendons on the contralateral, weight-bearing side.

Conclusions: Mechanical loading affected lubricin expression in flexor tendons, resulting in a 40% reduction of lubricin content, but these changes did not affect the gliding resistance of the flexor tendons.

Clinical Relevance: The gliding resistance of flexor tendons was not affected after a period of limited motion. This suggests that physical activity after a short period of limited motion does not lead to wear of intact tendons and their surrounding tissue.

Lubricin, also known as superficial zone protein and proteoglycan 4, was originally isolated from synovial fluid and identified as an essential lubricant in joints¹⁻³. Lubricin is synthesized and secreted selectively by chondrocytes in the superficial zone of articular cartilage^{4,5}. Subsequent studies have also found lubricin in tendons⁶⁻⁸, menisci^{8,9}, ligaments⁸, muscle⁸, skin⁸, and intervertebral discs¹⁰.

Lubricin exists more abundantly at or near the gliding surface of tissues, where these tissues are subjected to high levels of compressive and frictional forces. Lubricin is also present at the interface between collagen fiber bundles within a tendon^{6,11}.

The presence of lubricin in cells in particular load-bearing regions suggests that the synthesis of lubricin could be regulated by mechanical stimuli. Cyclic tension, but not hydrostatic pressure, significantly upregulates the expression of lubricin in chondrocytes that have been seeded in alginate constructs¹², whereas axial compression alone has no effect on the production of lubricin in chondrocyte-seeded three-dimensional scaffolds^{13,14}. However, surface motion, which includes both compressive and shear forces, upregulates the production of lubricin. Dynamic shear stimulates the biosynthesis of lubricin in bovine cartilage explants, and continuous passive motion

Disclosure: One or more of the authors received payments or services, either directly or indirectly (i.e., via his or her institution), from a third party in support of an aspect of this work. In addition, one or more of the authors, or his or her institution, has had a financial relationship, in the thirty-six months prior to submission of this work, with an entity in the biomedical arena that could be perceived to influence or have the potential to influence what is written in this work. No author has had any other relationships, or has engaged in any other activities, that could be perceived to influence or have the potential to influence what is written in this work. The complete **Disclosures of Potential Conflicts of Interest** submitted by authors are always provided with the online version of the article.

applied to whole joints stimulates biosynthesis of lubricin by chondrocytes^{15,16}.

The presence and function of lubricin at tissue surfaces support the paradigm that lubricin facilitates boundary lubrication at tissue surfaces that undergo relative motion. Deterioration of the boundary-lubricating ability of synovial fluid is associated with a decrease in lubricin concentration following injury or inflammatory arthritis¹⁷. Tribosupplementation with lubricin prevents cartilage degradation and restores chondroprotection¹⁸. Depletion of lubricin in flexor tendons results in increased friction between the flexor tendon and pulley¹⁹. The application of exogenous lubricin improves gliding of repaired flexor tendons and tendon grafts²⁰⁻²².

A number of studies have shown that mechanical loading regulates expression of lubricin by chondrocytes¹²⁻¹⁶. Little is known about the relationship between mechanical loading and lubricin expression in tenocytes or about the function of lubricin in flexor tendons (such as improving tendon gliding and preventing adhesion formation after immobilization following flexor tendon surgery). In this study, we examined the effect of stress deprivation on the expression of lubricin in flexor tendons in vivo. The gliding ability of flexor tendons with or without stress deprivation was also investigated.

Materials and Methods

Animals and Tendons

Forty-four flexor digitorum profundus tendons were harvested from the third and fourth digits of the forepaws of twelve adult mongrel dogs (ten to fifteen months old, 20 to 25 kg) that had been killed in the course of other studies^{23,24} approved by our Institutional Animal Care and Use Committee. As a part of those studies, which involved surgery on the second and fifth-digit tendons of one forepaw of each dog, the dogs were treated with a non-weight-bearing protocol, in which the operatively treated forepaw was splinted in wrist and elbow flexion and a sling was used to maintain the paw underneath the chest with a custom-made canine jacket for twenty-one days. Postoperative care included ten minutes of passive motion exercise of the paw and digits twice daily, seven days a week, to prevent joint contracture and adhesion formation in the operatively treated digits. The other forepaw of each dog moved freely, bearing full weight during ambulation.

The animals were killed at twenty-one days following surgery. Flexor digitorum profundus tendons were harvested from both the non-weight-bearing and weight-bearing forepaws. Four flexor digitorum profundus tendons harvested from the third or fourth digits of four forepaws of two dogs were used for immunohistochemistry, and twenty from the third digits of ten dogs were used to analyze lubricin gene expression with real-time RT-PCR (reverse transcription polymerase chain reaction). Twenty tendons from the fourth digits of ten dogs were first used for the evaluation of friction, and segments of the tendon were then used to quantify lubricin content by ELISA (enzyme-linked immunosorbent assay).

Real-Time RT-PCR

The expression of lubricin in the flexor tendons of the third digits was quantified with use of real-time RT-PCR. After each dog was killed, two 10-mm-long segments underneath the proximal pulley was harvested from the flexor tendons in the third digits of two forepaws. The tendon segment was stored at -80°C until the time of RNA extraction. The specimen was homogenized in TRIzol reagent (Invitrogen, Carlsbad, California) with a Mikro-Dismembrator (B. Braun Biotech International, Melsungen, Germany). Total RNA was extracted from the tendon segment according to the manufacturer's protocol. Contaminating genomic DNA was digested with DNase treatment (Roche Applied Science, Indianapolis, Indiana) and further removed with use of an RNeasy Mini Kit (QIAGEN, Valencia, California). The RNA concentration was determined with use of a RiboGreen RNA Quantitation Kit (Invitrogen). RNA was then reverse-transcribed into cDNA with use of a Tran-

Fig. 1

The experimental setup for measurement of friction between the tendon and proximal pulley. The weight was 500 g.

scriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Quantitative RT-PCR was performed with a LightCycler 1.5 (Roche Applied Science) to measure gene expression. The PCR primers, designed from canine-specific lubricin cDNA sequences, were 5'-GGCCCGCTATCAATTACC-3' and 5'-ACTTCATTATGGAGGAAACCTTTA-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference housekeeping gene. The primers for GAPDH were 5'-TATGATTCTACCCACGGCAA-3' and 5'-CAGTGGACTCCACAACATAC-3'.

Immunohistochemistry

Four forepaws were collected from two dogs. One flexor digitorum profundus tendon was harvested from the third or fourth digit of each paw. Each tendon was divided into two segments at the middle of the gliding region against the proximal pulley. One segment of tissue from each tendon was washed with saline solution. The other segment from each tendon was washed with saline solution and extracted with 2 M NaCl in PBS (phosphate-buffered saline) supplemented with 10 mM EDTA (ethylenediaminetetraacetic acid) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri) at 4°C for twenty-four hours. All tissue samples were embedded in Tissue-Tek O.C.T. (Optimal Cutting Temperature) compound (Sakura Finetek USA, Torrance, California) at -20°C and sectioned longitudinally at $7\text{-}\mu\text{m}$ thickness with a Leica CM 1850 cryostat. Immunohistochemical staining of lubricin was performed with use of a previously published protocol⁶. The primary antibody, anti-lubricin monoclonal antibody (MAb) S6.79²⁵, was applied at a concentration of $1\text{ }\mu\text{g/mL}$.

Friction Measurement

The friction between the flexor digitorum profundus tendon and the proximal pulley was measured as described previously^{26,27}. Briefly, the tendon and pulley were mounted on a testing device that consisted of one mechanical actuator with a linear potentiometer, two tensile load transducers, and a mechanical pulley (Fig. 1). The contact angle between the tendon and the proximal pulley was 50° . A 4.9-N load was used. The specimen was kept moist in a saline solution bath throughout the testing. Movement of the tendon toward the actuator was regarded as flexion. The actuator movement was then reversed, causing the tendon to move distally under the pull of the weight to simulate extension. The force differential between the proximal and distal tendon ends represented the gliding resistance. The gliding region in the testing was the region of the tendon that passes through the proximal pulley physiologically. The mechanical testing was preconditioned with three cycles of repetitive motion. The friction measurement obtained during the fourth cycle of motion was then recorded.

Lubricin Extraction and Quantification

After the friction measurement, the 10-mm-long segment designated for RNA extraction was dissected and weighed. The lubricin in each segment was extracted with 2 M NaCl in PBS supplemented with 10 mM EDTA and a protease inhibitor cocktail (Sigma-Aldrich) at 4°C for twenty-four hours.

A sandwich ELISA using peanut agglutinin (PNA; Sigma-Aldrich) and anti-lubricin MAb S6.79²⁵ was used to quantify lubricin. A high-binding ninety-six-well plate was coated for one day with PNA at a final concentration of $100\text{ }\mu\text{g/mL}$.

Fig. 2
Lubricin expression in flexor tendons in the loaded and suspended limbs. The error bars represent the standard deviation, and the asterisk indicates that the difference was significant. GAPDH = glyceraldehyde-3-phosphate dehydrogenase, which was used as a reference.

in 50 mM sodium bicarbonate buffer, pH 9.5. On the following day, serial dilutions of the lubricin extracts and of purified bovine lubricin, which was used as the standard, were incubated overnight on the PNA-coated plate at 4°C. The plate was subsequently washed with PBS plus 0.1% Tween-20. Nonspecific binding was blocked by addition of 1% BSA (bovine serum albumin) and 1% horse serum in PBS with 0.05% Tween-20 followed by incubation for two hours. Lubricin MAb S6.79 was subsequently added at a concentration of 0.1 µg/mL and incubated for sixty minutes at room temperature, followed by washing with PBS with 0.1% Tween-20. Anti-mouse IgG (immunoglobulin G) HRP (horseradish peroxidase)-linked whole antibody (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was added to the plate at a 1:2000 dilution and incubated for 60 minutes at room temperature. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) (TMB Substrate Kit; Pierce Biotechnology, Rockford, Illinois) was added. The

reaction was quenched with the addition of an equal volume of 2 M sulfuric acid. The absorbance was measured at 450 nm.

Statistical Analysis

Means and standard deviations for lubricin expression, lubricin concentration, and friction were calculated and reported. The comparisons between the paws subjected to stress deprivation and the control paws were analyzed with use of a paired t test. A p value of <0.05 was considered significant. All statistical analyses were performed with JMP software (version 8; SAS Institute, Cary, North Carolina).

Source of Funding

This study was supported by a grant from the Mayo Foundation and grants from the National Institutes of Health (RO1AR44391, RO1AR050180, R41AR057276, and P20RR024484).

Results

Deprivation of mechanical loading for twenty-one days resulted in a 40% reduction of lubricin expression in the flexor tendons in the suspended limbs compared with those in the contralateral, weight-bearing limbs ($p < 0.01$) (Fig. 2).

Lubricin was found primarily on the surface of the flexor tendons (Fig. 3). Sodium chloride extraction resulted in less intense lubricin staining in the flexor tendon of the suspended forepaw compared with the contralateral, loaded forepaw.

Consistent with the RT-PCR results, the flexor tendon in the suspended limb had significantly ($p < 0.01$) less lubricin, 1.6 ± 0.7 µg/mg, compared with the flexor tendon in the contralateral limb, 2.7 ± 0.6 µg/mg, as measured by ELISA (Fig. 4).

Although stress deprivation resulted in a decrease in lubricin expression and content of the non-weight-bearing

Fig. 3
Immunohistochemical sections showing the distribution and intensity of lubricin staining in flexor tendons, with and without sodium chloride extraction, in the loaded and suspended limbs. The scale bar is 50 µm.

Fig. 4

Fig. 4 Lubricin (Lu) content in flexor tendons in the loaded and suspended limbs. The error bars represent the standard deviation, and the asterisk indicates that the difference was significant. **Fig. 5** Gliding resistance of flexor tendons in the loaded and suspended limbs. The error bars represent the standard deviation.

Fig. 5

tendons, the gliding resistance did not change ($p = 0.50$) (Fig. 5). The gliding resistance of the weight-bearing flexor tendons was 58.7 ± 3.6 mN compared with 58.0 ± 3.5 mN in the limbs suspended for twenty-one days.

Discussion

Tendons primarily transfer tensile force between muscle and bone. Mechanical stress plays an important role in the development, degeneration, and regeneration of tendons²⁸. Limb suspension results in a decrease in the compressive and tensile forces applied to the canine flexor tendons. The expression of extracellular matrix proteins (such as aggrecan, collagen, decorin, and fibronectin) is significantly reduced in such tendons²⁹. In addition to the decrease in compressive and tensile forces, limb suspension results in a decrease in the extent of tendon gliding, since the suspended limb is not used for normal ambulation. In this study, we found that the expression and content of lubricin were significantly reduced in the flexor tendons in the suspended limb compared with those in the contralateral, weight-bearing limb. This is consistent with previous findings in articular cartilage, in which surface motion regulates the biosynthesis of lubricin^{15,16}. We conclude that lubricin may be a useful marker in the assessment of surface motion in tendons as well as in articular cartilage.

The friction between the flexor tendon and pulley is low, with a coefficient of friction similar to that of articular cartilage³⁰, and lubricin appears to play a lubricating role in both articular cartilage and flexor tendons^{1-3,19}. Elimination of lubricin by trypsin digestion significantly increases the gliding resistance of flexor tendons¹⁹. However, trypsin digestion simultaneously depletes both lubricin and other protein and nonprotein components, such as phospholipids and hyaluronic acid, that are believed to be involved in tendon lubrication¹⁹. In the present study, we further explored the lubrication mechanism in tendons. The expression and content of lubricin decreased significantly in the suspended flexor tendons, but the gliding resistance did not change. Similar results have been obtained in studies involving articular cartilage that compared wild-type, lubricin-null, and heterozygous mice. There was no *in vivo* difference in

friction between wild-type and heterozygous mice during short-term oscillation of loaded joints³¹. Another study showed that a threefold dilution of normal synovial fluid lubricated as effectively as normal synovial fluid³². The present study indicated the presence of an excess of lubricin in normal flexor tendons, as a loss of 40% of the lubricin was not sufficient to change the coefficient of friction of this tissue.

In addition to the physical function of lubrication, lubricin also possesses the biological ability to protect tissue surfaces^{33,34}, inhibits synovial cell overgrowth, limits the incorporation of separated tissues, and inhibits integrative tissue repair^{35,36}. The application of lubricin to the surface of a substrate impedes the attachment and proliferation of tenocytes³⁷. Digestion of lubricin on the surface of flexor tendons with trypsin facilitates cell growth on the tendon surface *in vitro*³⁸. Adhesion formation is a major complication after flexor tendon repair^{39,40}. The application of lubricin *in vivo* effectively decreases postoperative flexor tendon adhesions, but it has also been associated with the impairment of tendon-healing²⁴. The decrease in lubricin expression and content that was identified in canine flexor tendons after limited motion in the present study suggests a possible biological factor affecting adhesion formation after flexor tendon injury and repair. Specifically, the well-known tendency toward adhesion formation after such injuries may be exacerbated by an associated decrease in the synthesis of a known anti-adhesion factor, lubricin.

There are several limitations to this study. First, the surgery may have altered the normal activity and loading that the flexor tendons in the loaded forepaws underwent. We did not evaluate the routine activity of canine forepaws in this study. However, it was apparent that the loaded forepaw in each dog had more activity than the contralateral paw, which was suspended after it was surgically treated. Second, we did not determine if wear was enhanced following a reduction in lubricin content. Our previous studies showed that flexor tendons could maintain an intact surface during up to 1000 cycles of repetitive motion^{27,41}. This suggests that lubricin, a component of the flexor tendon surface, resists wear. In the present study, the

same washing procedure was used for each tendon that was designated for lubricin extraction, and the gliding resistance of the flexor tendons was the same in the two tendon groups. We believe that most of the lubricin remained on the tendon and the effect of washing was the same for all tendons. Third, the quantified lubricin content did not represent all of the lubricin in the flexor tendon segment. However, as the same experimental procedure was used for all tendons, the quantified lubricin content should be proportional to the total amount of lubricin in the flexor tendon segment. Fourth, local inflammation resulting from the surgery on the second and fifth tendons may have affected the mechanical properties or lubricin content of the tested tendons. However, the levels of messenger RNA for a number of growth factors in repaired tendons have previously been shown to return to normal levels within twenty-one days after tendon surgery^{42,43}. Therefore, we believe that the data obtained from the neighboring, intact tendon twenty-one days after surgery primarily reflect the activity level rather than the surgical intervention. Finally, lubricin was measured in the entire flexor tendon segment. We did not specifically assess lubricin on the gliding surface of the tendon and in the interior of the tendon separately.

This study furthers our knowledge of lubricin biosynthesis and function in tendons. Mechanical loading affected lubricin expression in tenocytes in a manner similar to that in chondrocytes. Also, lubricin expression and content could be reduced substantially without adversely affecting gliding resistance; this is again similar to the case in articular cartilage.

A short period of limited motion would therefore not increase the susceptibility of the intact tendons and the surrounding tissue to wear. ■

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