11-3-2006

The Effects of Cyclic Strain on Rat Tail Tenocytes

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A Thesis Submitted to the

Yale University School of Medicine

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

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2006

Abstract

THE EFFECTS OF CYCLIC STRAIN ON RAT TAIL TENOCYTES

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The purpose of this study was to examine the effects of cyclic tension on the expression of hyaluronic acid, its receptor (CD44), and total glycosaminoglycan content in tendon fibroblasts. An *in vitro* model was used to analyze tenocytes from the tail tendons of rats. Tenocytes in the experimental group were exposed to cyclic mechanical stress, and using ELISA, western blot, and colormetric dye-binding assays, the effect of strain on cultured tenocytes was examined.

Tenocytes exposed to mechanical strain produced 1528 ± 58 ng/mL (mean ± SEM) of hyaluronic acid, while those in a control environment produced only 730 ± 27 ng/mL; nearly a two-fold difference ($p<.0001$, $n=44$). CD44, the receptor for hyaluronic acid, was also detected in higher concentrations. Tenocytes under mechanical strain increased their concentration of CD44 by 62.5%, with tenocytes exposed to strain having an optical density of $26 \cdot 10^3 \pm 2 \cdot 10^3$ compared with $15 \cdot 10^3 \pm 1 \cdot 10^3$ in controls ($p<.05$, $n=6$). The total glycosaminoglycan content of the two groups did not differ significantly; strained cells produced 10.2 ± 0.6 µg/mL and controls producing 15.3 ± 3 µg/mL ($p=0.103$, $n=44$).

We conclude that mechanical strain in tendon fibroblasts is sufficient to induce the production of hyaluronic acid and increase the expression of its receptor, CD44. The results of our study suggest that the beneficial effects on tendon adhesion formation seen with both mechanical strain and hyaluronic acid may be related in their mechanism.

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Acknowledgements

I would like to thank my thesis advisor, J. Grant Thomson, MD, for his continued support, advice, and commitment during the course of this project. In the laboratory, Thomas McCarthy, PhD and Michael Centrella, PhD were an always-available and invaluable resource as I navigated the world of bench science.

Also, I want to express my gratitude to Martijn van Griensven, MD, PhD, Tanja Barkhausen, PhD, and Michael Skutek, MD, from Hannover, Germany who took the time to teach me the intricacies of tendon fibroblast tissue culture.

Finally, this work would not have been possible without the financial support of the Yale University Section of Plastic Surgery and the Office of Student Research. Thank you for investing in what I hope is a valuable contribution to our understanding of tendon biology.

Introduction

No subject has created more challenges, interest, or discussion in the field of hand surgery than flexor tendon injuries. Controversies on the proper approach to tendon surgery began as early as Galen and Avicenna, and by the 1930s, Bunnell had coined the term "No Man's Land" to describe a portion of the hand in which tendon injuries caused particular tribulation.(1)

The great difficulty with repairing flexor tendons surgically is the frequent creation of fibrotic adhesions between the tendon and surrounding tissues. These accumulations of scar tissue prevent the smooth gliding of the tendons, limiting movement and causing debilitating contractures that are the bane of hand surgeons.

The challenge presented by adhesions has been the focus of many research endeavors. Unfortunately, to date these have met with limited success. Are they truly a "biologic inevitability" as some authors(2) have described them?

Adhesions have long been an obstacle to proper healing after tendon injury or repair(3) and have many causes.(4) They differ in their degree of density, and different types of adhesions may be caused by independent regulatory pathways. This has led to attempts by some researchers to develop a standard methodology of measuring adhesion biomechanical strength.(5) Dense adhesions, for example, are characterized not only by an increased quantity of collagen than loose adhesions, but also with an increase in the ratio of type III to type I collagen and their ratio of cross-linking.(6) In tendons,

fibroblasts from the fibro-osseous sheath have biochemical and cellular differences which may enable them to migrate and contribute to their role in adhesion formation.(7)

Many techniques have been developed to mitigate the development of adhesions in tendons including: enveloping the repaired tendons within silicone sheathes(8), fibrin glues(9, 10), iontophoresis(11), pulsed electromagnetic fields(12, 13), and thermal preconditioning to induce the formation of cytoprotective heat shock proteins(14, 15). The results have not translated into successful clinical applications.

A number of biochemical agents have also been used to thwart the formation of adhesions. Non-steroidal anti-inflammatory agents that inhibit cyclooxygenase (COX) and in turn, prostaglandin synthesis, have received some attention. Indomethacin has been shown to be beneficial at reducing adhesions(16-19), and some work shows that ibuprofen can also reduce adhesions in both oral(20) and injectable(21) form. Conversely, prostaglandins are known to cause or facilitate adhesion formation.(22) Corticosteroids(23, 24) and promethazine(25, 26) have also been seen to exert a favorable effect. Some of the benefits to pharmacological anti-inflammatory therapy may be due to the decreased edema in tissues. This is supported by biomechanical animal studies which examine the time course of gliding function after injury and reveal a significant impediment to gliding within hours of injury, long before adhesion formation can occur.(27)

Intraoperative exposure of repaired tendons to 5-fluorouracil has been shown to diminish adhesion formation in rabbit(28, 29) and chicken(30) models, as well as reducing the level of TGFβ1 (thought to be involved in the formation of adhesions).(29) Later research has shown that this does not decrease the strength of the tendon repair.(31)

This may be due to its inhibitory effects on matrix metalloproteinases (particularly mmp2 and 9).(32) Aprotinin, another protease inhibitor, has similarly been shown to be effective in reducing adhesion formation(33) as has halofuginone, an inhibitor of collagen I transcription(34) and Beta-aminopropionitrile (BAPN), an inhibitor of collagen maturation.(35) The effect of these chemicals is to hinder collagen production, a needed component for the creation of adhesions. Collagen itself, when added topically to injured tendons, induces the upregulation of collagenase to mediate these effects.(36-38)

Although some of these have shown promise, the data are usually limited to small case series or animal models with clinical trials often yielding disappointing results. Despite some indications to the contrary(39, 40), physical barriers to adhesion formation such as PTFE have also not proven a useful technique(41-45). The use of an anti-adhesive membrane (ADCON-T/N) was evaluated in human flexor tendon repair in a prospective double-blind randomized control trial (RCT), and total active motion was evaluated at 3, 6, and 12 months after the repair. Although the length of time taken to achieve the final range of motion was shorter in treated patients, the total range of motion achieved was not improved.(46) The failure of ADCON-T/N to prevent adhesions has been supported by other researchers.(47)

As a whole, these studies emphasize that there are a variety of factors which may influence the formation of adhesions after tendon surgery. Along with these biochemical signals, mechanical strain also seems to be important for the proper functioning and healing of tendons. The tension-bearing property of tendons is important for the normal mechanics of joint motion. Unfortunately, after surgery tendon motion is limited by pain and rupture rates can be high if motion is allowed. Depending on the particular repair

technique and mobilization protocol, the rupture rates of digital flexor tendons range from 4%(48) to 46%(49) and 17% for thumbs(50). On average, the rupture rate approaches 10%(48, 50-65), and the rate of "excellent" or "good" results (using Strickland's revised scoring technique, a commonly used tool to evaluate outcomes of hand surgery(53)) is often below 75%, although individual results vary considerably.

Historically, hand immobilization was used to protect flexor tendons after repair. This period often lasted three or more weeks, and while effective at minimizing the risk of tendon rupture, it had the disadvantage of maximizing the formation of adhesions. More recently, a substantial body of literature describing the beneficial role of early hand mobilization has been published. We now know that mobilization is beneficial to the smooth gliding of tendons and prevention of adhesions. And although active mobilization can result in rupture(66) or gap formation(67) in animal models, most data suggest a positive role. Overall, animal models using active rehabilitation mobilization protocols have been shown to decrease adhesion formation without undue risk of rupture when compared to immobilization.(68) This translates into repairs which are stronger, have a more efficient healing process(69) and greater range of motion(70) in the joints that they affect.

The timing of when tendon mobilization is started after surgery is also an important factor. In a study that examined canine flexor tendons after surgical repair, immediate mobilization was superior to delayed mobilization in returning range of motion (a 50% increase) and dramatically better than immobilized tendons (more than a five-fold improvement in angular rotation).(71) This benefit has been corroborated by other studies.(72)

There is still some discussion on which protocols maximize the beneficial effects of mechanical strain while introducing the least risk of rupture. It is known that several parameters play a role in determining outcome. The frequency of motion, for example, is an important factor, with increased frequency correlated with improved tensile strength.(73) A prospective multi-center RCT was performed to examine the effects of daily passive motion and whether increased duration of motion could improve tendon gliding after flexor tendon repair. The authors report that there was a significant benefit to increased duration of controlled motion on the range of motion, increasing the angle of rotation by 19 degrees.(74)

This has also translated into clinical research(74-76), and it is now clinically accepted practice to apply these protocols to patient care as the most effective method of preventing adhesions(4) even in the presence of concurrent digital nerve injuries.(77)

Previously, it was thought that active mobilization protocols (involving the contraction of muscles in continuity with the repaired tendons) would lead to unacceptable rupture rates. Mobilization however, both active and passive, helps tendon healing and decreases adhesion formation. Animal studies have supported a benefit to tendon strength and smooth gliding by using controlled passive movement(78) as well as active movement. Prospective trials in humans have shown an advantage in patients who have passive(79) and active(57-59) post-operative mobilization. Although some authors report an advantage to active motion(62), at least some data suggest that neither is superior to the other.(49) Active motion can result in higher rupture rates, but an analysis of different protocols shows that the risk can be comparable to passive motion if

performed judiciously(63) and if the patient avoids imprudent acts which account for half of all tendon ruptures.(50)

Cyclic mechanical loading of tendons to mimic strains encountered during mobilization protocols reveals that tenorraphy failure rates may depend on the repair technique used.(80) Indeed, there is an entire body of literature which examines the surgical techniques of tendon repair(42) which will not be discussed at length here except to say that after tendon injury or repair, there is an increase in the friction of tendon gliding which remodels with time.(81) High friction suture techniques(82) and many other factors(83) may centrally contribute to adhesion formation and possibly rupture.

Fibroblasts show a response to mechanical strain *in vitro* at both a cellular and biochemical level.(84) Tendons *in* vivo exposed to extracorporeal shock waves after injury have been shown to have an increased number of capillaries and decreased formation of adhesions, with a correspondingly higher force required to cause rupture.(85)

Cyclical mechanical strain, in particular, seems to be important. When exposed to cyclic mechanical strain, flexor tendons fibroblasts proliferate and align in the direction of tension to form a thicker growth layer.(86) Constant strain, however, appears to be of lesser benefit in healing tendons.(87, 88)

One question that exists regarding the effect of mechanical strain on healing tendons is the degree to which strain actually contributes to the improvements seen with active and passive motion rehabilitation protocols. One theory might argue that only motion is important, as the primary mechanism is the mechanical separation of fibrotic

bridges which are putative adhesions. Another view is that the strain is primarily important, as the host of growth factors regulated by and affecting strain certainly plays a role.

The question was addressed by a study which examined *in vivo* chicken flexor tendons. Motion and strain each had an independent roll in generating tensile strength, and the combination of the two was greater than either alone. An increase in collagen production, however, was seen only when tendons experienced some type of strain.(89) Other studies have shown that the degree of excursion (change in joint angle) is of minimal importance in determining the extent of adhesion formation or whether a repair will have good range of motion.(90)

The effect of mechanical strain on the growth factor milieu of tendons is paramount. After tendon injury or repair, a host of growth factors are upregulated. Microarray analysis of rat flexor tendons under strain demonstrate an "antifibrotic" pattern of gene regulation, with decreased transcription of collagen I and III, FGF, PDGF, and IGF1.(91) IGF has been shown to promote fibroblast growth in culture.(92-94) In contrast, TGFβ1 (which is often associated with increased adhesion formation) was found to be upregulated during strain in this study.(91) The import of this is somewhat unclear.

The regulation of the TGFβ pathway seems to be particularly important in the pathogenesis of fibrosis and formation of adhesions. It has been shown to be associated with adhesion formation, and some studies show that the addition of TGFβ to tenocytes *in vitro* can increase collagen production(95), while neutralizing antibodies to TGFβ can decrease collagen production(96). The role of individual isoforms of TGFβ (TGFB1, 2, and 3) are not yet clear. Most evidence points to TGFβ1 acting to contribute to adhesion

formation, and less so for TGFβ2 and 3. This has been supported by research showing decreased adhesion formation in the presence of TGFβ1 blocking antibodies(97) and the observation that although TGFβ1 is expressed in healthy tenocytes, the levels rise sharply after tendon injury.(98)

Some research suggests that the site of action of individual growth factors varies significantly. In one study which examined the effect of a tendon injury on growth factor regulation at ten days after injury, TGFβ was detected around the repair site and just proximal to it, while other factors such as VEGF and PDGF were expressed throughout the tendon. Some growth factors such as EGF, IGF, and bFGF were not seen in tenocytes; only in pro-inflammatory cells in the repair site.(99) This increase in TGFβ activation is most pronounced in the tendon sheath and epitenon, peaking within two weeks of injury or repair.(100)

The expression of VEGF in injured tendons suggests that the supply of nutrients and oxygen may be an important factor in determining the adhesion forming response of healing tendons. When tendons are examined after repair, progressive changes in the microvasculature can be observed.(101) Revascularization of tendons following injury occurs progressively over a 7-21 day window, forming an extrinsic vascular supply.(102) In one study, the authors noted a decreasing density and more longitudinal orientation of vessels associated with an increasing range of motion after the acute healing phase of the first 21 days had passed.(103)

Other vascular signaling intermediaries have been implicated as well. Nitric oxide synthase (NOS) levels double after an acute tendon injury, and appear to be integral to the repair process. Blocking NOS production creates a persistent, chronic

inflammatory response that leads to an increased incidence and severity of adhesions.(104) Lactate can also modulate early wound healing and can stimulate collagen production after injury. Cultured tendon cells exposed to lactate have been shown to increase collagen production(105), suggesting that lactate levels may directly modulate collagen production during tendon wound healing or repair.

As newer molecular biology techniques become available, they will advance our understanding of the complex growth factor regulation that plays a critical role in the biology of adhesion formation and tendon healing. Adenovirus mediated gene transfer appears to be an efficient, dose dependent method of delivering transgenes to flexor tendons and at viral concentrations of less than 10^9, does not appear to engender a significant inflammatory response.(106) Also, antisense oligonucleotides to procollagen have been shown effective at reducing collagen production in human tenocytes.(107)

In sum, many applications for the prevention of adhesions after tendon repair have been tried. Unfortunately, few have met with clinical success. To understand the biological basis of adhesions, a closer look at tendon biology is warranted.

Tendon is specialized connective tissue specifically designed to withstand tension and allow the smooth gliding of tissues. On gross examination, tendons themselves are bright white cord or band-like structures that form the structural connections between bone and muscle. Water constitutes approximately 70% of tendon mass(108) with collagen I accounting for the majority of the dry mass. Tendons contain additional structural elements, including bone attachment and insertion sites, blood vessels, and a dense extracellular matrix. The primary role of tendons is structural, which is reflected in

their decreased metabolic activity. Oxygen consumption, for example, is 7.5 fold lower when compared with skeletal muscles.(109) This property allows them to function in relatively ischemic environments; an important adaptation as tendons are commonly in compact anatomical regions with limited tissue perfusion and often under enormous tension which would otherwise cause tissue necrosis. However, the decreased metabolic rates also contribute to slower healing after a traumatic insult.(110)

Although tendons contain some elastin, they are comprised predominantly of thick collagen type I fibers and spindle-shaped tenocytes interspersed in a columnar fashion among the fibers. The glycosaminoglycan hyaluronic acid (hyaluronan, hyaluronate) is present in small quantities. External to the collagen fibers is a layer of paratenon fibroblasts. The internal fibroblast may be responsible for the bulk of the collagen synthesis, whereas the external cells may provide lubrication for tendon gliding during motion.(111)

At a micro-structural level, tendons are formed by the successive grouping of larger and larger bundles of fibers. At the smallest level, a triple-helix polypeptide chain forms "primary bundles" of tropocollagen which are only 15 angstroms in diameter. Several small fibers of tropocollagen together form a 35 angstrom microfibril, many of which unite to form a subfibril of 100-200 angstroms in width. Subfibril groups form 500-5000 angstrom fibrils, and eventually the 50-300 μm fascicles of tendons.(112)

Each bundle of tendon collagen is covered by an *endotenon.* The septa of these endotenon form together to constitute an external *epitenon* that covers the three-dimensional surface of the tendon. This is further complemented in the hand by a thin adventitia known as the *paratenon* within a synovial-like fluid environment.(42)

This arrangement is coupled by a complex waveform or crimp structure of different topographies that forms a dynamic rope-like tendon capable of tremendous tensile strength. Biomechanical analyses, for example, reveal that tendons can withstand forces equivalent to thousands of Newtons.(113) The capacity to produce such a robust, complex structure lies in the equally intricate arrangement of cellular subtypes.

Tendons contain several types of cells. Tenocytes (or tendon fibroblasts) account for 90 to 95% of tendon cells. Synovial cells, chondrocytes located near osseous insertions sites, endothelial cells, smooth muscle cells of arterioles, and immunologic cells form the remainder. Collectively, they are responsible for the formation, maintenance and interactions of the extracellular matrix.

Along with the previously mentioned cellular components, extracellular fluid, and collagen and elastin fibers, the extracellular matrix (ECM) contains a fourth component – the ground substance. Ground substance is the portion of the extracellular matrix comprised of glycoproteins and proteoglycans – large molecules which are formed by the union of a glycosaminoglycan through a trisaccharide link to a protein core. Glycosaminoglycans (or GAGs) are synonymous with “mucopolysaccharides” and exist independently in the extracellular matrix as the most plentiful heteropolysaccharides in the body. GAGs are formed as long polymers of modified disaccharides and uronic acid. Because of their large size (molecular weights in the tens of millions) and predilection for absorbing water, glycosaminoglycans often play a structural role in lubrication or minimizing mechanical compression.

One glycosaminoglycan of particular interest is hyaluronic acid (HA). Hyaluronic acid is a high molecular weight glycosaminoglycan first described in 1934.(114) It is formed by the repeating disaccharide N-acetylglucosamine and D-glucuronate.(115) Because of its high molecular weight, random coiled structure, and ability to attract water, hyaluronic acid is hydrodynamic and forms solutions that have high viscosity, elasticity, and propensity for filtration.(114)

Hyaluronic acid is unique in that it is not sulfated and not covalently bonded to a protein, though it does complex with other proteoglycans. It lubricates tendon tissues and is thought to play an important role in the early stages of connective tissue healing and scarless fetal wound healing.(116-119) In an experimental model involving fetal lambs, tendons which were injured healed with no subcutaneous scaring, no adhesions, and regained a smooth gliding surface with well organized collagen architecture, although adult sheep controls experienced significant dense scarring and adhesions.(120)

Interestingly, it has been shown that acellular amnion membrane (which contains elevated concentrations of hyaluronic acid(121)) can prevent the adhesion of tendons after repair without compromising tendon healing.(122, 123) In one study, the combined effect of amniotic membranes applied in conjunction with hyaluronic acid was examined. Although the researchers reported that this was greater than either amniotic membrane or hyaluronic acid alone, it did not reach statistical significance.(124) Other studies, however, have shown in animal models that topical amniotic fluid alone applied during tendon repair is sufficient to both decrease adhesion formation and improve the tensile strength of the repair.(125)

In animal models of osteoarthritis, the addition of hyaluronic acid has been shown to provide a lubricating effect, decreasing the friction of tissues.(126) Even in healthy joints, the lubricating effect of hyaluronic acid can be seen.(127)

It has been suggested that the molecular weight of hyaluronic acid plays an important role in determining its effect on adhesion formation.(128) Some investigators have attempted to examine whether the molecular weight affects the mechanical friction in joints. In one study, the researchers injected 1% hyaluronic acid of two different molecular weights into the joint spaces of rabbits and determined that molecular weight did not play a role in determining the lubricating characteristics.(129) Unfortunately, the molecular weights chosen by the researchers were both similar (one and two million Daltons) and relatively low. As a consequence, they may not have been sufficiently different to allow for detection of lubricating or signaling potential. Moreover, it may not be the lubricating role of hyaluronic acid that is central to its effects on tendon healing.

Hyaluronic acid was previously thought to be an inert molecule that provided mechanical compression support in connective tissues. It is now understood that hyaluronan is an active ligand that exerts important regulatory and healing effects through hyaluronan-binding proteins, also known as hyaladherins.(114) These interactions make hyaluronic acid an important player in regulating cell mobility, adhesion, and proliferation; all important cellular functions in tendons. Hyaluronic acid is also present intracellularly and may be incorporated from extracellular stores(130), or produced *de novo* from within the cell(131) where it is thought to influence cell proliferation and motility.(114)

CD44, the primary receptor for hyaluronic acid(132), was first cloned in 1989.(133) Antibody mediated activation of the CD44 receptor produces an anti-inflammatory response(134) and organisms which are engineered to eliminate CD44 gene expression (-/-) are unable to properly respond to injury.(114) In addition, hyaluronic acid and its primary receptor, CD44, have been implicated as important cell adhesion mediators in the pathogenesis of cancer(135).

Of the many roles hyaluronic acid is thought to have, the most germane to tendons is its role as a structural lubricant and metabolite in the regulation of cell proliferation and adhesion formation. Applied topically during flexor tendon repair in animal models, hyaluronic acid has been shown to decrease adhesions at the repair site and synovial sheath. In a study involving the flexor tendons in dogs, for example, hyaluronic acid was applied topically to the second and fifth flexor tendons which had been transected and repaired. After five weeks of immobilization, the tendons exposed to hyaluronic acid had a significant decrease in both gross and histological adhesions.(136) This has also been seen in other studies of rabbits.(137)

After flexor tendon repair, canine tendons exposed to a hyaluronic acid-containing solution showed reduced excursion resistance.(138, 139) By decreasing the friction and force on the repaired tendon, increased gliding occurs, which decreases adhesion formation(140) and the likelihood of rupture.

Multiple *in vitro* and *in vivo* animal studies have supported a role for hyaluronic acid at preventing adhesions.(141, 142) Collagen contents (adhesions) in peri-tendon tissues are decreased, smooth gliding is improved(143), and range of motion is greater.(144) Hyaluronic acid has been shown to increase expression of VEGF and

collagen IV when applied to healing tendons after repair, both potential anti-adhesive mediators.(140)

Although some studies have not shown hyaluronic acid to decrease adhesion formation(145-147), they are in the minority. During tendon repair in a rabbit model, Seprafilm, a bioresorbable membrane containing hyaluronic acid and carboxymethyl cellulose, worked as well as hyaluronic acid gel in the prevention of adhesions and improvement of range of motion when compared to controls who received no hyaluronic acid.(148) Benefits are also observed for tenolysis of existing adhesions in a chicken model(149) and in dogs.(136) Other preparations of hyaluronic acid such as Healon and viscoelastic gel have been shown to be effective at reducing adhesion formation, even when injected into zone II flexor tendon repairs in immobilized primates.(150) The addition of a phospholipid lubricant, dipalmitoyl phosphatidylcholine, has also been shown to augment the action of hyaluronic acid, decreasing the friction coefficient and formation of adhesions in rabbit flexor tendons.(151) Exogenously applied hyaluronic acid has also been shown to inhibit tendon fibroblast proliferation involved in the formation of adhesions in rabbit models.(152)

A potential role for hyaluronic acid to mitigate adhesion formation is of particular interest in hand surgery as adhesions can cause significant functional impairment after surgical tendon repair. Hyaluronic acid, although effective at reducing adhesions experimentally, has been disappointing clinically.(153, 154)

One of the challenges of applying hyaluronic acid as a therapy is its rapid absorption in the body. Hyaluronic acid can be degraded within a matter of hours, and within 24 hours, significant dilution may take place. Higher molecular weight

preparations and those that contain a higher initial concentration of hyaluronic acid experience a slower decline.(155, 156) This is further supported by evidence showing that hyaluronic acid which has been modified to persist longer has a beneficial effect. One group of researchers using a chicken model mixed hyaluronate with carboxymethylcellulose and prevented the formation of dense adhesions.(157)

A rabbit study which reported a beneficial effect from hyaluronic acid, decreasing adhesion formation from 95% to 45%, also noted that when adhesions did form in the presence of hyaluronic acid, they were less severe.(158) The authors show in a later study that improved function may have been a result of increased viscosity of the hyaluronic acid preparation.(159) Using *ex vivo* canine tendons as a model, a derivatized form of hyaluronic acid, which through chemical modification persists longer than native hyaluronate, maintained a lower gliding resistance over many repeated cycles of tendon movement than hyaluronic acid or saline.(82)

Clinically, some researchers have reported beneficial results with hyaluronic acid(160), but the vast majority have not.(42) This disparity between the very promising *in vitro* and animal study findings, and the disappointing clinical observations, is an area of great interest. A better understanding of the biology and regulation of hyaluronic acid is needed.

It has been shown that removing the tensional loading from tendons either experimentally or surgically changes matrix characteristics including increases in total glycosaminoglycan content.(111) Conversely, adding motion to repaired tendons has been shown to be beneficial to tendon healing, resulting in fewer adhesions, increased strength of repair, and more longitudinal alignment of collagen bundles compared with

healing tendons without movement.(54, 71) How tendon cells perceive changes in tensional loading, however, is not well understood.(161)

While the effects of tensional loading on a variety of protein expression products in tendon are known(159-168), the effect of mechanical strain on hyaluronic acid production and its receptor, CD44, is uncharacterized in tendons. Do these two important mediators of adhesion formation and tendon healing share a common pathway?

There is some evidence that suggest they might. In fibrocartilage tissue, mechanical strain *has* been shown to increase hyaluronic acid production and several of its upstream mediators.(162) If tendon fibroblasts react in a similar way, it would support the view that the beneficial effects of mechanical strain, the cornerstone of current clinical management of tendon healing and adhesion prevention, are acting at least in part through hyaluronic acid, arguably the most promising biochemical player in the prevention of adhesions. With a better understanding of the biochemistry of hyaluronic acid and mechanical strain, a more elegant solution to the problem of adhesions may one day be in hand.

Purpose

The objective of this study was to isolate rat tendon fibroblasts (tenocytes) and to investigate the effect of cyclical mechanical strain on matrix component production, specifically hyaluronic acid (HA), its receptor (CD44) and total glycosaminoglycan (GAG) production.

Materials and Methods

Note on individual performance of research tasks:

All actual labor for the project including, but not limited to, all procedures, cell culture work, animal cell and tissue harvesting, all methods and experiments, generation of data, analysis of data, production of reagents, design and selection of assay systems, use of assay systems including ELISA, western blots, and colormetric dye-binding assays, preparation of cell lysates, and antibody utilization were performed personally by Richard Crockett. In addition, Richard Crockett was personally responsible for the use, configuration, and repair of the FlexCell unit, as well as the selection, evaluation, procurement and price negotiation of all required reagents and materials, budget management, and project timeline planning.

Certain elements of the research relied on personnel other than Richard Crockett:
Training of Richard Crockett in the use of western blot technique and photographic film use was performed by Dr. Michael Centrella.

Training of Richard Crockett in surgical dissection techniques for rat tail tendons was performed by Dr. J. Grant Thomson.

Training of Richard Crockett in the culture of tenocytes was performed by Dr. Martijn van Griensven in Hannover, Germany.

Certain elements of the conceptual and pragmatic research plan relied heavily on the creative input of personnel other than Richard Crockett:

Development of laboratory strategies to efficiently obtain data involved discussions with Dr. Thomas McCarthy and Dr. Michael Centrella.

Development of strategies involved in design of the strain protocol and overall research goals involved discussions with Dr. J. Grant Thomson.

Methods Summary:

The research involved several stages, which are explained in detail below. Briefly, rat tail tendons were surgically dissected and the tendon fibroblasts were removed and cultured. After two passages, these cells were transferred to flexible membrane culture dishes and assigned to an experimental or control group. The experimental group was exposed to a cyclical mechanical strain, while the control (or static) group was not. The cells were then analyzed for changes in three targets of interest: hyaluronic acid, the receptor for hyaluronic acid (CD44), and total glycosaminoglycan content.

Harvest of Tendon Cells:

The tendon fascicles were harvested from 12 adult Sprague-Dawley rats (Charles River Laboratory; Wilmington, MA). All animal care complied with the IACUC and NIH protocols for the care and use of laboratory animals. The rats were sacrificed with an overdose of isofluorane and cervical dislocation. The tail and hindquarters were

immersed in 95% ethanol then thoroughly rinsed in sterile saline. After transecting the tail, the skin was removed and discarded. The transected tail was transferred to a 150mm Petri dish (Corning; Corning, NY) and using sterile technique, tendon fascicles from dorsal, ventral, and lateral tendon groups in the anterior two-thirds of the tail were removed. After the paratenon was removed, the tissue was divided into 3-5mm^2 samples and placed in 60mm Petri dishes (Corning). For each piece of tissue, a small area of the dish was abraded with a number 11 scalpel blade. The tissue was allowed to partially air dry for several minutes in a sterile laminar flow hood in order to increase adherence. 3-5mL of culture medium was then slowly added. The culture medium consisted of Dulbecco's modified Eagle's Medium (DMEM) with Ham's F-12 (1:1) and L-glutamine (Gibco; Grand Island, NY) supplemented with 10% fetal calf serum (Gibco). Penicillin/streptomycin species (100 U/mL) was also added to the medium.

Culture of Tendon Cells:

In a 37°C non-moving 5% CO_2 incubator, the tissue was left undisturbed for 2-3 days. Tenocytes migrated from the clumps of tendon tissue. After tenocyte growth reached 75-80% confluence, the tendon tissue was removed. The medium was removed by suction and the dish was bathed in 1-2mL 0.25% trypsin with EDTA (Gibco) at 37°C for 10 minutes. The trypsonized cells were placed in a 15 mL Falcon tube to which culture medium was added. The cells were then collected by sedimentation, resuspended in culture medium and plated in the culture medium on 25cm^2 filter-top culture flasks (Nunc; Roskilde, Denmark). The cells were observed daily under an inverted phase-contrast microscope (Olympus; Melville, NY). Medium was changed every other day.

When the cells had reached 75-80% confluence, the medium was discarded and the cells were freed with trypsin as described above. Cells were then split and plated onto three 80cm^2 filter-top culture flasks. When these cells had reached 75-80% confluence, they were plated on Bioflex® plates (Flexcell International; McKeesport, PA); culture dishes containing a flexible silastic membrane bottom. These culture dishes contained 6 wells, with each well bottom consisting of a flexible membrane which had been previously coated with fibronectin to facilitate fibroblast cell adhesion.

Cultured cells were plated on the same type of Bioflex plate, irrespective of group (strained or static). Only after the cells had been cultured and were ready for the strain protocol were the culture dishes assigned in a random fashion to either the strained or static group. This was done to prevent any selection bias based on cell line. Cell density was determined using a hemocytometer and cells were plated at approximately 10,000 cells/57.75cm² Bioflex plate.

Strain Protocol:

The Flexercell® Strain Unit applies cyclic strain to cells cultured to confluence on flexible membranes (Flexcell International). The unit exerts a vacuum pressure on a silastic membrane at the bottom of each 6-well plate that has been pre-coated with fibronectin. The unit fits inside a standard CO_2 incubator and the duration, frequency, and magnitude of stress can be adjusted. A 5% change in the size of the membrane approximates a 2% change in the length of cells on the membranes; similar to the amount of elongation collagen experiences under physiologic conditions.(161) To select an appropriate strain regimen, we attempted to mimic active rehabilitation protocols, which

involve periods of repeated strain followed by comparatively longer periods of rest and recovery. Previous studies have shown that the magnitude and duration of strain(163), as well as the length of the rest between stain periods are important parameters(164), but the frequency of strain is less important.(163) Accordingly, cells were strained at 1 Hz for five minutes and allowed to rest for 55 minutes. This was repeated over the course of 8 hours, after which the cells were allowed to rest for 24 hours before collection so that any translational or post-translational events would have sufficient time to occur. Both the medium and cells were collected for analysis.

Cultured tenocytes were divided into two groups – an experimental group, which was exposed to the mechanical strain regimen, and a control or static group which was not exposed to any strain. Both groups were extracted and cultured in identical conditions at the same time and each replicate was derived from an independent cell lineage. During the strain regimen, strain cells which had been cultured on the Bioflex 6-well plates were placed in the strain unit. Static cells were placed in the same incubator at the same time as the strained cells but were not exposed to any strain.

Hyaluronic acid content

Hyaluronic acid concentration was measured using an enzyme linked protein assay (Corgenix; Westminster, CO). Using a capture molecule called Hyaluronic Acid Binding Protein (HABP) which is bound to horseradish peroxidase (HRP), the assay is visualized with a chromogenic substrate and measured in optical density with a spectrophotometer. Because hyaluronic acid is primarily secreted into the extracellular

tissue matrix, we compared the concentration in the media from strained cells to that found in the media of static cells.

CD44 receptor expression

Immunolabelling for CD44 was performed using a polyclonal rabbit antibody to CD44 (Santa Cruz Biotechnology; Santa Cruz, CA). After the medium had been removed from the Bioflex plates, the cellular layer was collected and homogenized using a sonicator. The homogenate was normalized for total protein content and separated by charge to mass ratio using SDS polyacrylamide gel electrophoresis. The proteins were electroblotted onto PolyScreen polyvinylidene difluoride transfer membrane (PerkinElmer Life Sciences, Wellesley, MA). The western blot was then probed using primary antibody (as described above) in TBST with milk at 20°C overnight. The secondary antibody was goat, anti-rabbit conjugated to HRP (Santa Cruz) and immunocomplex formation was visualized with chemiluminescence and exposure to photographic film.

Total glycosaminoglycan (GAG) content

To measure the amount of glycosaminoglycan (GAG) present in the medium, a colormetric assay was used which involves the binding of a dye (dimethyl-methylene blue) to GAGs (Biocolor Assays; Newtownabbey, Northern Ireland). A dye solution which includes DMB dye and an inorganic solvent are added to the sample. The dye is in excess and binds with GAGs, precipitating out of solution. After centrifugation, the resulting pellet contains the dye-GAG complex, and the supernatant contains unbound

DMB dye and solvent. Resuspension of the pellet in propanol to disassociate the dye from the GAGs allows the concentration of solublized dye to be read on a spectrophotometer.

Data Analysis

An unpaired two-tailed Student's t-test was used to compare strained and static groups. A value of $p < 0.05$ was considered significant. Western blots were optically scanned and analyzed using ImageJ (NIH; Bethesda, MD).

Results

During the harvest of the tendon fibroblasts, 12 animals were dissected and the tendons were removed. Two additional animals were used solely for practice of the tissue harvest and culture and were not part of the experiment. Although it was initially challenging to separate the tendon from the surrounding tissue (including the paratenon), once the technical elements were refined the process became straightforward. Each animal yielded a sufficient quantity of tendon tissue such that each control and experimental cell line consisted of tenocytes with a distinct cell lineage from the others. These were cultured in parallel, under identical conditions. One culture developed a fungal infection before assignment to either the experimental or control group and was discarded.

Hyaluronic Acid Content

Since hyaluronic acid is produced by fibroblasts and found primarily in the extracellular environment, the medium in which the cells were cultured was analyzed quantitatively for hyaluronic acid content. The removed media were normalized for total protein content before analysis. Using an ELISA assay, 44 medium samples (22 experimental, 22 control) were analyzed. The medium of tenocytes which had experienced cyclical mechanical strain had a greater concentration of hyaluronic acid (1528 ± 58 ng/mL (mean ± SEM)) compared with the medium of tenocytes that had not been subject to strain (730 ± 27 ng/mL). This constitutes nearly a two fold increase in hyaluronic acid content ($p < .0001$). (See Table 1).

Table 1

	Assay		
Group	Hyaluronic Acid *ng/mL*	CD44 Receptor *O.D.*	Glycosaminoglycans *µg/mL*
Strained	1528 +/- 58	25525 +/- 2461	10.2 +/- 0.6
Static	793 +/- 27	15454 +/- 1443	15.3 +/- 3.0
p value	< .0001	0.024	0.103

Comparisons between strained and static cells for hyaluronic acid, CD44, and glycosaminoglycan content. Values are mean ± standard error of the mean.

CD44 receptor expression

Hyaluronic acid as a signaling molecule acts primarily through its receptor, CD44. To assess whether there were changes in the population of the CD44 receptor, the cell membranes of the tenocytes were analyzed for the presence of CD44. To allow for the transcription and membrane translocation of the receptors, a delay of 24 hours between the end of the mechanical strain regimen and collection was instituted. Using a western blot analysis, 6 (3 experimental, 3 control) cellular homogenate samples from independent cell lineages were analyzed for expression of CD44. (See figure 1). Tenocytes under mechanical strain increased their concentration of CD44 by 62.5%, with tenocytes exposed to strain having an optical density of $26 \cdot 10^3 \pm 2 \cdot 10^3$ compared with $15 \cdot 10^3 \pm 1 \cdot 10^3$ in controls ($p<.05$). (See table 1).

Figure 1

Inset of western blot for CD44. Lanes 1 through 3 represent the increased signal in experimental cells exposed to cyclic strain compared to control lanes 4 through 6 which shows CD44 production in static cells.

Total glycosaminoglycan content

The total amount of glycosaminoglycan produced by tenocytes in both the strained and static groups was measured to examine what effect mechanical strain had on the production of these structural polysaccharides. Using a commercially available colormetric dye-binding assay, 44 medium samples (22 experimental, 22 control) were analyzed for content of glycosaminoglycans. No significant differences were detected in glycosaminoglycan content between the media of tenocytes which had been cyclically strained versus cells which were not, with strained cells producing 10.2 ± 0.6 μg/mL and controls producing 15.3 ± 3.0 μg/mL ($p = 0.103$). (See Table 1).

Discussion

Proper tendon healing after repair requires a balance between appropriate fibrous connective tissue formation to provide tensile strength and the formation of excessive scar tissue, fibrosis, which impedes the smooth gliding of structures and leads to adhesions and impairment of function. Mechanical strain has been shown important in this collagen production and the linear organization of collagen fibrils(54). Its role both in animal models and clinical settings is widely accepted. The active rehabilitation protocols used for hand surgery patients currently employed are designed to tread between these two extremes: too little activity or strain can result in the formation of adhesions, while excessive strain can result in rupture at the site of tenorraphy.

Understanding the effects of mechanical strain on tendon fibroblasts is important in finding adjunct medical therapies which would mitigate fibrosis and adhesion formation without weakening tendon repair. One such candidate is hyaluronic acid—a high molecular weight polysaccharide found throughout the body that provides lubrication in joints and the smooth gliding of tendons. More recently, research has shown that in addition to its structural and lubricating role, hyaluronic acid as an important signaling molecule for cell motility, adhesion, and proliferation.

Hyaluronic acid has been shown to be beneficial in preventing or decreasing adhesions in animal models including chicken(142, 144, 149), rabbit(137, 143, 148, 152, 158), canine(136), horse(141), and primate.(150) Unfortunately, this success in animal models has not translated into clinical applications.(42, 153, 154) This disappointing gap suggests that a closer look at hyaluronic acid may be warranted.

Our study examined whether mechanical stress might directly influence hyaluronic acid regulation. The model we chose was one in which rat tail tenocytes were exposed to cyclic strain *in vitro*. One question that this raises is whether this is a valid model for examining the effects on tendon fibroblasts.

Dissociated cells and tendon explants do appear to be good models for examining the effects of mechanical strain on tenocytes. Dissociated tenocytes bond well to fibronectin as demonstrated in biomaterial studies which examine adhesion strength.(165) Tendon explants and dissociated fibroblasts grown in culture retain their ability to respond to mechanical stress.(111, 161, 166-171) Moreover, the effects seen correlate well with the effects of mechanical strain seen *in vivo*.(172-176)

Part of the difficulty in teasing apart the role of hyaluronic acid is that it seems to behave differently based on its molecular weight, concentration, and the presence of other growth factors. In fact, despite evidence that hyaluronic acid is a key molecule in tissue healing, cell migration, and adhesion formation, we know little about how it is regulated.

Probably the most important regulator of hyaluronic acid is hyaluronic acid synthase (HAS), particularly the second isoform, Has2(177, 178). In a study that examined wound healing with cell lines that expressed sense and anti-sense Has2, the increased cell migration and faster healing seen with sense Has2 was absent in cells expressing anti-sense Has2. The anti-sense Has2 cells were also associated with an increase in adhesive plaques. Exogenous hyaluronate was not able to overcome the deficiency, suggesting that activation of Has2, more than the quantity of hyaluronic acid, is the key determiner of how adhesions are formed and regulated by the hyaluronan pathway.(179)

This raises the question: if hyaluronic acid is beneficial to tendon healing, would inhibiting the enzymes which degrade it (hyaluronidases) be beneficial as well? The answer in tendons has not been explored. In intra-peritoneal adhesions however, where some data suggest that hyaluronic acid is helpful in reducing the adhesion formation(180), the effect has been examined(181, 182) and some researchers have seen a benefit.(183)

In sum, it seems that hyaluronic acid has more than one role as a signaling molecule. At a molecular level, hyaluronidase can suppress the rapid interactions that occur early in cell adhesion formation. When a small amount of hyaluronic acid exists, it can serve to facilitate cell-matrix adhesion, whereas excess hyaluronic acid strongly inhibits cell adhesion.(184) Thus, it seems that hyaluronic acid may help to allow cells to interact, move and bind together, while at the same time, larger amounts may help prevent adhesion formation.

This study showed a significant increase in both hyaluronic acid and its receptor (CD44) when tenocytes were exposed to cyclical mechanical strain *in vitro*. Our study is the first to examine the effects of mechanical strain on the regulation of hyaluronic acid in tendons. It is clear from our study that mechanical strain, which is an integral part of the repair process, plays a role in the regulation of tendon fibroblast hyaluronic acid production. How that translates into adhesion formation still needs to be explored, however.

When we examined the total glycosaminoglycan content, it was not found to be significantly altered by mechanical strain, though other investigators have shown previously that many stimuli including *in vitro* culture alone can alter total and sulphated

GAG content(111). The increase in hyaluronic acid, but not total glycosaminoglycan content, may reflect the preferential effect of mechanical strain on the hyaluronan pathway. Another possibility is that we did not observe any change in glycosaminoglycan content in response to mechanical strain because of limitations of the assay, which performed poorly even during calibration of standards. Additionally, the assay we performed examined the presence of glycosaminoglycans in the supernatant. The predominance of glycosaminoglycans may have been intimately associated with the cell membranes and not readily present in the supernatant.

Hyaluronic acid is a promising target for mitigating adhesion formation. Unfortunately, the clinical applications to date related to post-operative tendon repair have been disappointing(153, 154). In addition to the complexities of its regulation and mechanisms of action, use of hyaluronic acid has been challenged by its rapid catabolism and dilution in the body.

Newer, derivatized formulations of HA show longer persistence and less friction in tendons after repair(185) and have been used for a variety of purposes(186) from coating metallic artificial joints(187) to forming biologically compatible hydrogels.(188) Other researchers have created cross-linked hyaluronic acid films which contain DNA encoding hyaluronan synthase 2 (HAS2). The goal of these biofilms is to provide a physical barrier while at the same time delivering hyaluronan and upregulating HAS. The films are subject to the same catabolism by hyaluronidases as native hyaluronic acid, and as such subject to breakdown, but cross-linked hyaluronic acid-collagen sheets which are resistant to hyaluronidase over several weeks have been developed.(189) Hopefully, these promising discoveries will translate into viable clinical applications.

The future role of hyaluronic acid as an adjunct medical therapy to prevent the formation of adhesions after tendon surgery is unclear. The current challenges in understanding how hyaluronic acid fits into the larger process of wound healing are interesting avenues of research, however, and as the details of its regulation and mechanisms of action are revealed, perhaps a clearer path to preventing tendon adhesions will be as well.

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