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THE MECHANOTRANSDUCTION RESPONSE
OF TENDON CELLS TO TENSILE LOADING

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MICHAEL LAVAGNINO

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Roger C. Haut
Major Professor's Signature

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THE MECHANOTRANSDUCTION RESPONSE OF TENDON CELLS TO TENSILE LOADING

By

Michael Lavagnino

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ABSTRACT

THE MECHANOTRANSDUCTION RESPONSE
OF TENDON CELLS TO TENSILE LOADING

By

Michael Lavagnino

The ability of tendon cells to sense and respond to load is central to the concept of mechanotransduction and the maintenance of tendon homeostasis. Tendon cells sense load through a mechano-electrochemical sensory system(s) that detects mechanical load signals through the deformation of the cellular membrane and/or the cytoskeleton. This cellular deformation produces tension in the cytoskeleton, which can be sensed by the cell nucleus through a mechano-sensory tensegrity system to elicit a metabolic response. While the precise level (magnitude, frequency/rate, and duration) of mechanobiological stimulation required to maintain normal tendon homeostasis is not currently known, it is very likely that an abnormal level(s) of stimulation may play a role in the etiopathogenesis of tendinopathy. Although tendinopathy has been well described pathologically, the precise etiopathogenesis of this condition remains unsettled. Classically, the etiology of tendinopathy has been linked to the performance of repetitive activities (so-called overuse injuries). This has led many investigators to suggest that it is the mechanobiologic over-stimulation of tendon cells from repetitive loading that is the initial stimulus for the degradative processes that have been shown to accompany tendinopathy. Although several studies have been able to demonstrate that the *in vitro* over-stimulation of tendon cells in monolayer can result in a pattern(s) of gene expression seen in clinical cases of tendinopathy the strain magnitudes and durations used in these *in vitro* studies, as well as the model systems, may not be clinically relevant. Using an *in*

vitro rat tail tendon model, the objective of this research was to study the mechanobiologic response of tendon cells *in situ* (within their normal extracellular matrix), to various tensile loading regimes. The studies have shown that the gene response of tendon cells to load is both frequency and amplitude dependent and that tendon cells appear to be "programmed" to sense a certain level of stress. Model analyses combined with the experimental results have demonstrated that both strain rate and strain amplitude are able to independently alter rat interstitial collagenase gene expression through increases in fluid-flow-induced shear stress and matrix-induced cell deformation respectively. The studies have also shown that the absence of stress has a profound effect on the catabolic response of tendon cells, which in turn decreases the mechanical properties of the tendon independent of its collagen fiber distribution. The studies have shown that isolated fibrillar damage can occur within tendons and produce a localized upregulation of interstitial collagenase in response to altered (decreased) tendon cell stimulation. This weakens the tendon and may put more of the extracellular matrix at risk for further damage with subsequent loading. From these studies the hypothesis is forwarded that the etiopathogenic stimulus for the degenerative cascade that precedes the overt pathologic development of tendinopathy is the catabolic response of tendon cells to mechanobiologic *under-stimulation* as a result of microscopic damage to the collagen fibers of the tendon. This dissertation is a collection of research involving the response of tendon cells to changing load conditions and the examination of the implications of these responses as a potential etiopathogenic mechanism for the onset of tendinopathy.

DEDICATION

To my joy, my beloved, my wife, Nicole.

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INTRODUCTION

Tendinopathy, a syndrome of tendon pain, localized tenderness, and impaired performance, is a common and major health issue in workers and athletes who perform repetitive activities (Renstrom and Woo 2007). Although the pathology of tendinopathy has been well described, the precise etiopathogenesis of this condition remains unsettled (Renstrom and Woo 2007). Classically, the etiology of tendinopathy has been linked to the performance of repetitive activities (overuse injuries) (Almekinders et al. 1993). A proposed algorithm for the onset of overuse tendinopathy involves altered cell-matrix interactions in response to repetitive loading (Archambault et al. 1995). In this scenario, repeated strains below the injury threshold of the tendon induce degenerative changes in the tendon-matrix composition and organization (Jarvinen et al. 1997; Jones et al. 2006; Jozsa and Kannus 1997). The degeneration of the extracellular matrix leads to a transient weakness of the tissue making it more susceptible to damage from continued loading. This damage then accumulates until the overt pathology of tendinopathy develops (Archambault et al. 1995). While this is a feasible algorithm for the development of overuse tendinopathy, the precise mechanism(s) which lead to altered cell-matrix interactions have not been described. To better understand the mechanical association between tendon matrix and tendon cells, it is necessary to understand the following: 1) the composition of the tendon extracellular matrix, 2) the contribution(s) of these extracellular components in defining the material properties of the tendon, 3) the mechanisms by which mechanical signals are transmitted through the extracellular matrix to the tendon cells, and 4) the cellular response to these mechanical signals.

Tendon composition

Tendons are described as soft connective tissues which link bone to muscle and consist of solid (collagen fibers, cells, and matrix constituents of proteoglycans and glycoproteins) and fluid phases (Kannus 2000; Riley 2005; Woo et al. 1997). Type I collagen is the main component of the solid phase (65-80% dry weight) and is organized within the tendon in parallel fiber bundles with hierarchies of fibrillar arrangement down to microfibril size (Kannus 2000; Kastelic et al. 1978) (Figure 1). Under polarized light microscopy, tendon collagen fibrils appear in a sinusoidal wave pattern referred to as crimp (Diamant et al. 1972). Collagen has the ability to form covalent intramolecular and intermolecular cross-links, which are the keys to its tensile strength characteristics and resistance to chemical or enzymatic breakdown (Tanzer 1973; Woo et al. 1997). The matrix, or ground substance that surrounds the collagen, consists of proteoglycans, glycosaminoglycans (GAGs), and glycoproteins. Proteoglycans and GAGs only make up a small percentage of the total dry tendon weight, but due to their highly negative charge, these molecules attract and limit the movement of water, which represents 60-80% of the total wet weight (Woo et al. 1997). This water binding capacity improves the mechanical properties of tendon against shear and compressive forces (Kannus 2000). Glycoproteins in tendons include both structural and adhesive molecules. The structural glycoproteins (elastin, fibrillin) provide elastic properties of tendon while the adhesive glycoproteins mediate cell-matrix interactions (e.g. tenascin-C, fibronectin, thrombospondin)(Riley 2005). Each tendon component (collagen, crosslinks, crimp, matrix, water) has mechanical significance and together they form a well-organized tissue for optimal load distribution and response (Woo et al. 1997).

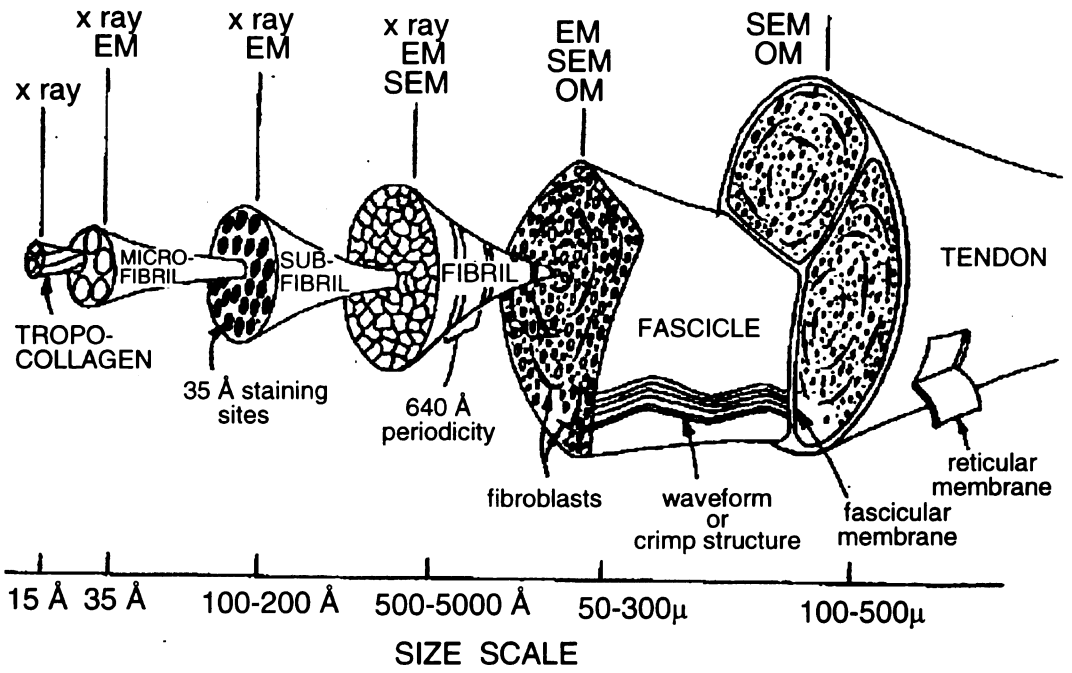

Figure 1 The hierarchical collagen network of tendon (Kastelic et al. 1978).

Tendon Computational Models

Based on these structural components (collagen fibers, proteoglycan matrix, and fluid) computational models of tendon have been created to further understand the mechanical response of tendon to applied strain. Although no models have examined the role of tendon mechanical load on cell deformation, many structural and continuum models exist that accurately model overall tendon mechanical behavior.

Collagen Fibers

Initial structural and microstructural models investigated parameters (orientation, number, distribution) of the collagen fibril, the main solid constituent (70-80% dry weight) of tendon, that relate to tissue morphology to describe the mechanical function of the tendon (Belkoff and Haut 1992; Comninou and Yannas 1976; Diamant et al. 1972; Kastelic et al. 1980; Kwan and Woo 1989; Lanir 1983; Stouffer et al. 1985; Viidik 1972). These models assumed that the toe-in region occurs due to a structural feature of the tissue, i.e., the gradual removal of crimp during deformation leads to increasing stiffness. The earliest structural model described tendon as a cable consisting of these crimped strands of microfibrils where the stress behavior is determined by the properties of the individual strands (Diamant et al. 1972). These microfibrils were modeled as having elastic segments joined by rigid hinges based on the elastica problem in mechanics (Figure 2).

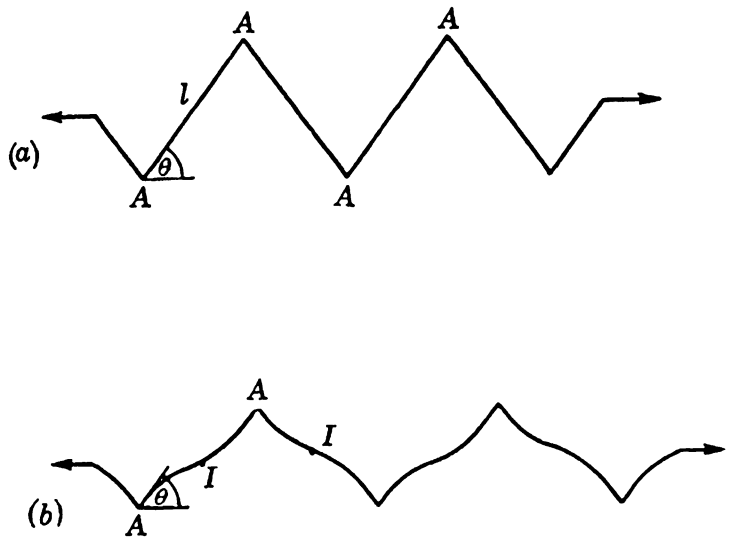

Figure 2 Extension of an elastic collagen fiber zig-zag crimp with apex points of infinite rigidity (Diamant et al. 1972).

Investigators advanced this model to explain additional structural features of crimp including the disappearance of crimp apices and the variability in crimp angle along the depth and length of the tendon using linearly elastic collagen fibers (Comninou and Yannas 1976; Kastelic et al. 1980; Stouffer et al. 1985) (Figure 3).

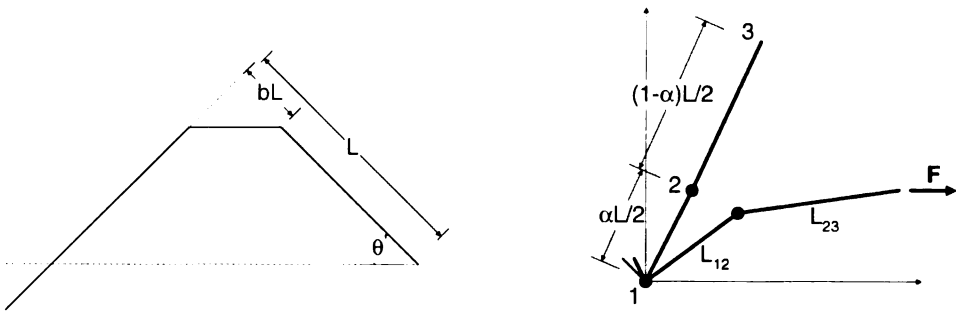

Figure 3 Advancements in crimp definition using a blunted zig-zag (b: crimp blunting factor) used in the SSL model (Kastelic et al. 1980). The undeformed and deformed configurations of collagen crimp with spring apex points (Stouffer et al. 1985).

Including crimp angle variations throughout the tendon was first incorporated in the sequential straightening and loading (SSL) model of collagen crimp (Kastelic et al. 1980). This model suggested that crimped fibrils are assumed to have a negligible resistance to extension while loaded fibrils resist deformation through a linear elastic relationship, thus resistance arises only from the elasticity of already straightened fibrils (Kastelic et al. 1980). This distribution of fibril angle in the tendon leads to a sequential fibril straightening and loading that result in the initial nonlinearity of the toe region (Kastelic et al. 1980). Investigators than described crimp using a modified SSL model, with varying initial fiber lengths of a bilinear elastic collagen fiber that have a specified

failure stretch/strain (Figure 4) (Belkoff and Haut 1992; Crisco and Panjabi 1996; Hurschler et al. 1997; Kwan and Woo 1989; Liao and Belkoff 1999).

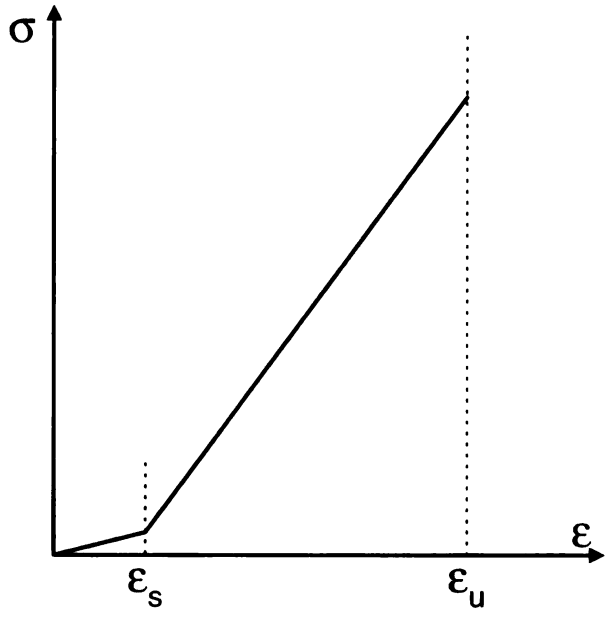

Figure 4 A schematic diagram showing the bilinear stress-strain curve for a single collagen fibril. Fibrils can vary by strain at which the crimp is fully stretched (S) or at which ultimate failure occurs (U) (Kwan and Woo 1989).

These models defined varying initial fibril lengths and varying failure lengths either arbitrarily (Kwan and Woo 1989), based on normal distributions (Crisco and Panjabi 1996), or extensively on the organization of the collagen microstructure (fiber density and distribution) (Hurschler et al. 1997) to determine how collagen affects the material properties. Other studies have further refined the model by using a quasi-linear viscoelastity law incorporating a relaxation function and a nonlinear time independent elastic response for collagen fibers (Decraemer et al. 1980; Frisen et al. 1969; Fung 1967; Haut and Little 1972; Lanir 1980; Viidik and Ekholm 1968). One proposed viscoelastic model, the single integral finite strain (SIFS), utilizes the concept of constitutive

branching to allow different constitutive equations at different elongations and the model is fully nonlinear and reduces to classic viscoelasticity (Mooney-Rivlin) if linearized (Johnson et al. 1996). Thus instead of a bilinear elastic model to describe crimp as shown previously (Kwan and Woo 1989), this model describes the change in micromechanism caused by the onset or cessation of collagen fiber recruitment as the tendon is extended as a change in constitutive equations. This model appears very accurate at modeling stress-relaxation, peak cyclic stresses, and stress strain curves (Johnson et al. 1996).

Proteoglycan Matrix

Although these models accurately depict the whole range of tissue behavior from initial load to failure, by only taking into account collagen structure and properties they lack potential mechanical properties from the remainder of the tendon constituents (extrafibrillar matrix and water). Tendons have been previously modeled as a composite material to incorporate both collagen fiber and matrix properties (Ault and Hoffman 1992; Ault and Hoffman 1992; Luo et al. 1998; Puxkandl et al. 2002; Redaelli et al. 2003; Scott 2003; Wilson et al. 1997; Wren and Carter 1998). One such composite model, similar to the pure collagen model proposed by Hurschler (Hurschler et al. 1997), included both extensive fiber and matrix properties (Wren and Carter 1998). This model allowed for uncrimping, stretching, and breaking of collagen fibers, but in addition described the fibers ability to rotate in the matrix as fully, partially, or un- constrained. Another researcher investigated the relationship between the matrix and the fibers and the likelihood of potential "links" between fibers (Wilson et al. 1997). This model utilized a plane stress finite element model with nonlinear spring elements (fibers) overlaid on the continuum elements (matrix) to study the possibility of shear stress within the tissue

(Figure 5) (Wilson et al. 1997). The non-linear springs provide the model with the ability to simulate the nonlinear behavior attributed to the crimp of the collagen fibers.

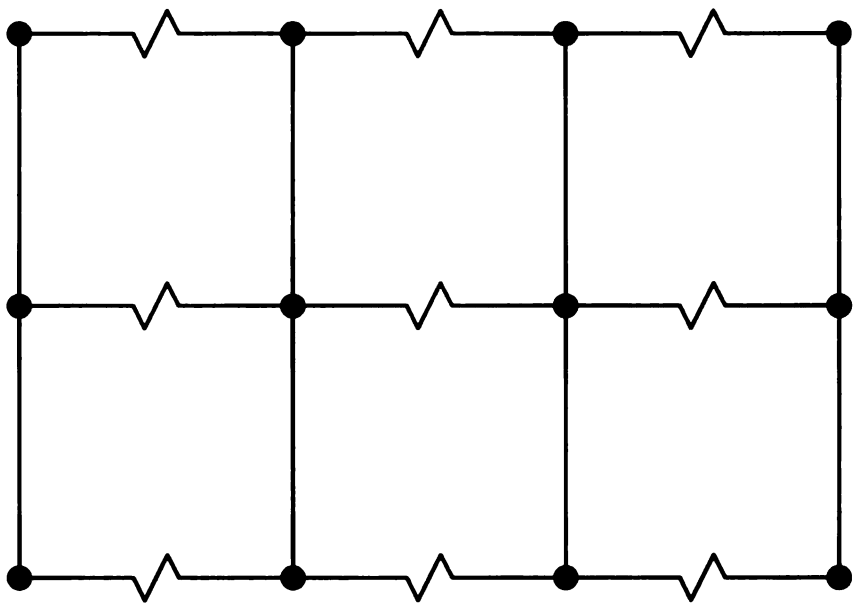

Figure 5 Spring-element model showing nonlinear spring elements (fibers) overlaid on the continuum elements (matrix) (Wilson et al. 1997).

This model determined that shear transfer may occur from linking between collagen fibers, but unfortunately only small deformations were modeled. The idea that a model should include fiber-fiber and fiber proteoglycan interaction began a subset of models that delve into modeling collagen fibril crosslinks from the molecular standpoint of matrix components, most notably glycosaminoglycan (GAG) chains. A sliding GAG filament model was proposed in which the GAG chain can slide and re-engage and thus provide mechanical stability (Scott 2003). These GAG chains, located on proteoglycans play a role in retaining water as well as creating a bond between collagen fibrils. One model incorporated this fiber-matrix interaction as a composite material with collagen

fibrils embedded in a proteoglycan rich matrix, where the matrix is mostly loaded under shear (Figure 6a) (Puxkandl et al. 2002). Deformation in the tendon occurs through extensibility of collagen fibrils and nonfibril (crosslink, matrix) deformation. The composite structure was modeled in series with collagen fibril and proteoglycan matrix as viscoelastic elements taking into account molecular friction, cross links, viscous relaxation, and matrix shearing (Figure 6b) (Puxkandl et al. 2002).

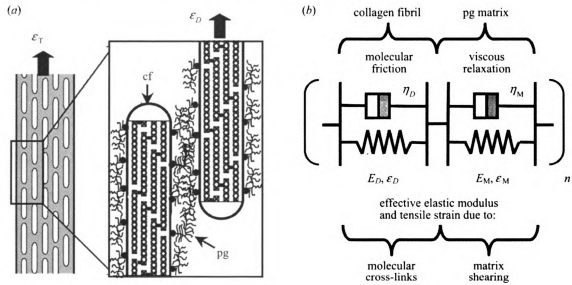

Figure 6 (*a*) Schematic representation of the hierarchical structure of a collagen tendon. If (a fibre of) the tendon is stretched by an amount ε_T, this is distributed between the collagen fibrils (cf) with a tensile strain ε_D and the proteoglycan-rich matrix (pg), which is mainly sheared. Covalent cross-links between molecules are drawn schematically within the collagen fibrils. (*b*) An illustration representing a mechanical model, where fibrils and matrix are considered as viscoelastic systems arranged in series. E_D is the elastic modulus of the fibrils that depends critically on the covalent cross-links. η_D is the viscosity of the fibrils, possibly due to friction between molecules. E_M is the effective elastic modulus of the matrix and η_M is the viscosity of the matrix (Puxkandl et al. 2002).

A similar model of proteoglycan links was also proposed with 40% of elongation due to fibril length change, and the remainder suggested due to relative movement of fibrils (Redaelli et al. 2003). Shear stress was assumed responsible for force transfer from fibril

to fibril. Fibril length, diameter, and interfibrillar distance were used as variables. Using this model, it was found that a stress-transfer matrix with low elastic modulus was sufficient and an increase in collagen fibril diameter alone cannot explain tendon mechanical properties (Redaelli et al. 2003).

Interstitial fluid flow

None of the previously mentioned collagen or collagen-matrix structural models include the permeability of the tendon or look at fluid exudation (Hannafin and Arnoczky 1994) and its role in the stress strain response (Haut and Haut 1997) within the tendon. A general 3D viscoelastic model for fibrous tissue was proposed that takes into account fluid flow, fiber density, and fiber orientation (Lanir 1983). The collagen fibers were assumed to only be subjected to uniaxial strain along the length of the fiber, with no compressive strength and the fluid flow through the matrix was due to a hydrostatic pressure differential (Lanir 1983). The model accurately predicted that the unfolding of the fiber leads to increased hydrostatic pressure or fluid flow through the matrix (Lanir 1983). Multiple continuum theories include tendon permeability and interstitial fluid flow in tendon models (Adeeb et al. 2004; Atkinson et al. 1997; Butler et al. 1997; Chen and Ingber 1999; Yin and Elliott 2004). These continuum models have added the structural morphology of the tendon extracellular matrix with fluid using a biphasic approach (Atkinson et al. 1997; Giori et al. 1993; Luo et al. 1998; Wakabayashi et al. 2003; Yin and Elliott 2004). One such biphasic model assumed that tendon was transversely isotropic in the fiber direction and the fibers are assumed to be piecewise linear elastic (Yin and Elliott 2004). Each of these models has predicted the importance of fluid flow and permeability in determining intertendonous strains and the exudation of

fluid flow from the tendon. These theories however do not take into account the nonhomogeneity of the crimp pattern (Hansen et al. 2002) nor the viscoelastic properties of the collagen fibers (Haut and Little 1972).

Collagen, matrix, and fluid flow

There are relatively few tendon models that take into account collagen structure, and a porous matrix that allows for fluid flow. One finite element model assumed the collagen crimp as an outer helical arrangement of 62° inclination (Figure 7) (Atkinson et al. 1997). The central core was then modeled as nonlinear poroelastic material with varying permeabilities (Atkinson et al. 1997). This model predicted fluid exudation and fiber straightening by having the outer collagen fibers wring out the fluid containing central portion.

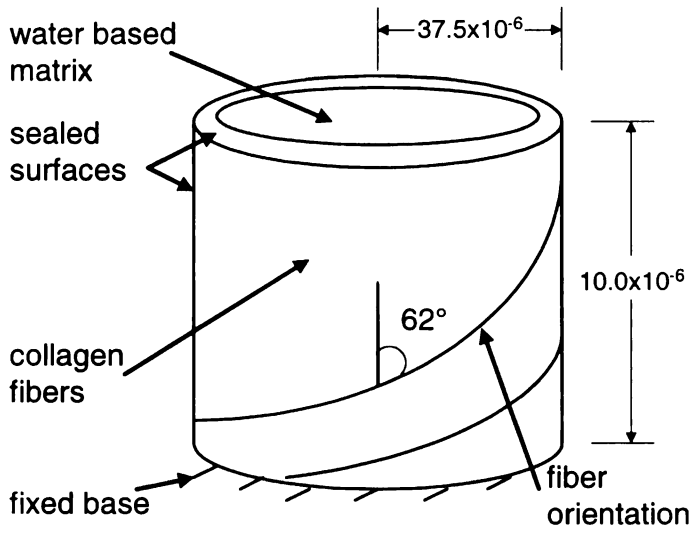

Figure 7 Conceptualization of the geometry of a fascicle used as the basis of a finite element model (Atkinson et al. 1997).

Another model examining the role of fluid in tissue and potential causes of fluid exudation addressed three mechanisms: 1) crimp straightening, 2) Poisson's ratio – determined by cross links, and 3) osmotic pressure (Adeeb et al. 2004). Another

investigator used a finite element system to track changes in fluid pressure and collagen orientation to examine the alterations in tendon composition with mechanical load (Giori et al. 1993). The maintenance and rearrangement of the tendon's fibrous extracellular matrix was associated with regions where stretching and distortion of cells take place as the tendon was physiologically loaded (tendon wrapped around bone). The tendon was considered to be a two-phase linear elastic fiber-reinforced composite defined using the rebar formulation of the ABAQUS FEM code (Figure 8) (Giori et al. 1993). This model assumed the tendon had no crimp initially (Giori et al. 1993). Results from this study showed that the hydrostatic stress and distortional strain are important tissue level mechanical stimuli regulating the composition of connective tissue and gene expression (Giori et al. 1993).

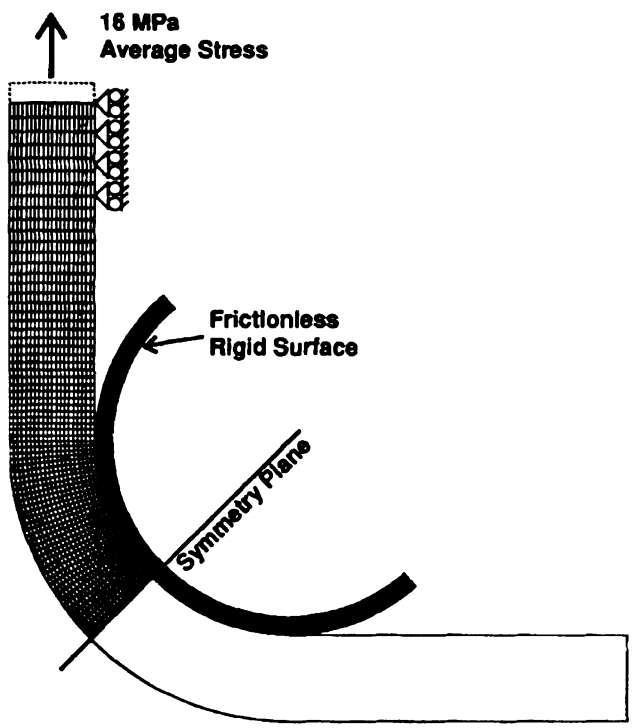

Figure 8 A finite-element model of tendon as a two-phase linear elastic fiber reinforced composite using the rebar formulation of ABAQUS (Giori et al. 1993).

Other biphasic tissue studies have suggested the importance of both the fluid and solid phases in the transmission of strain from the tissue to the cell, or mechanotransduction (Baer et al. 2003; Guilak and Mow 2000). Currently there are no tendon models that model the mechanical response of tendon strain using the solid and fluid components of tendon and then take that response to determine what is happening on the cellular level. Although each component is known to play an important role in the mechanical response of tendon to load, their influence on cellular mechanotransduction, or the transmission of strain through the extracellular matrix to the cell, within the tendon is unknown.

Cell-Matrix Interactions

The ability of tendon cells to sense and respond to a physical stress with a biological response, the concept of mechanotransduction, is vital in maintaining tendon homeostasis (Banes et al. 1995; Ingber 1997; Wang and Ingber 1994). Tendon cells sense physical stress through a mechano-biological sensory system(s) that detects mechanical load signals through the deformation of the cellular membrane and/or the cytoskeleton (Adams 1992; Banes et al. 1995; Brown et al. 1998; Ingber 1997; Wang 2006; Wang et al. 1993; Wang and Ingber 1994; Watson 1991). Cell membrane deformations may open or close stretch-activated ion channels, which control the influx of second-messenger molecules such as calcium and inositol triphosphate (IP_3) (Banes et al. 1995; Sachs 1988; Sachs 1988; Shirakura et al. 1995). These second messengers can activate a wide array of cellular machinery including DNA synthesis, mitosis, cell differentiation, and gene expression (Binderman et al. 1984; Ryan 1989; Sachs 1988; Sachs 1988). Cellular deformation also alters the cytoskeletal tension, which in turn, can be sensed by the cell nucleus through a mechano-sensory tensegrity system to elicit a

metabolic response (Adams 1992; Arnoczky et al. 2002; Banes et al. 1995; Ben-Ze'ev 1991; Ingber 1997; Wang 2006; Wang et al. 1993; Wang and Ingber 1994; Watson 1991). For both of these signaling pathways, change in cell shape with applied stress is thought to occur through the binding of the cell to extracellular matrix proteins such as collagen and fibronectin (Banes et al. 1995; Rosales et al. 1995; Sung et al. 1996). The binding between the extracellular matrix proteins and the interior cytoskeleton of the cell occurs through the integrin family of cell surface receptors (Banes et al. 1995; Ingber 1991; Janmey 1998; Rosales et al. 1995; Shyy and Chien 1997; Sung et al. 1996; Wang et al. 1993). Thus mechanotransduction, in response to tendon load, is likely mediated through the deformation of the extracellular matrix, which in turn would result in *in situ* cell deformation (Banes et al. 1995; Sachs 1988; Watson 1991). The results of a recent study support the hypothesis that mechanical loads placed on tendons result in a concomitant *in situ* deformation of the cell nucleus (Arnoczky et al. 2002). As has been proposed in cartilage, this nuclear deformation may play a significant role in the mechanotransduction of these tissue loads into intracellular signals (Guilak 1995; Guilak and Mow 2000; Guilak et al. 1995).

Mechanotransduction, Homeostasis, and Pathology

The importance of load (stress) in the homeostasis of connective tissues has been well documented (Akeson et al. 1974; Hannafin et al. 1995; Noyes 1977; Woo et al. 1982; Woo et al. 1997; Yasuda and Hayashi 1999). Several studies have shown that stress deprivation in ligaments and tendons results in significant alterations in their structural and functional properties (Akeson et al. 1974; Hannafin et al. 1995; Noyes 1977; Woo et al. 1982; Woo et al. 1997; Yasuda and Hayashi 1999). While these

alterations in the extra-cellular matrix appear to be cell mediated, the exact mechanism by which tissue load (or lack thereof) affects this process is unclear. Numerous *in vitro* investigations have demonstrated that when a deformable substrate on which adherent cells have been cultured is cyclically strained this extrinsic deformation activates a wide array of cellular machinery including DNA synthesis, mitosis, gene expression, and cell differentiation (Almekinders et al. 1993; Banes et al. 1994; Banes et al. 1995; Banes et al. 1995; Bhargava et al. 1999; Binderman et al. 1984; Birukov et al. 1995; Brighton et al. 1991; Brown 2000; Cheng et al. 1996; Hsieh et al. 2000; Matyas et al. 1995; Sumpio et al. 1990). Understanding the relationship between tendon strain and cell deformation may provide insight into the role of physical stress in the homeostasis of normal tissue. While the precise level (magnitude, frequency, and duration) of mechanobiological stimulation required to maintain normal tendon homeostasis in not currently known, it is very likely that an abnormal level(s) of stimulation may play a role in the etiopathogenesis of tendinopathy (Archambault et al. 1995).

Over-stimulation

Several investigators have suggested that tendinopathy is initiated by repetitive loading which over-stimulates the tendon cells leading to a mechano-biologic response of degeneration (Archambault et al. 2002; Archambault et al. 2001; Skutek et al. 2001; Tsuzaki et al. 2003; Wang et al. 2003). Over-stimulation of tendon cells *in vitro* has been shown to induce increases in inflammatory cytokines and degenerative enzymes (Almekinders et al. 1993; Archambault et al. 2002; Banes et al. 1999; Banes et al. 1995; Tsuzaki et al. 2003; Wang et al. 2003). The majority of these *in vitro* studies are based on the response of large numbers of tendon cells cultured on artificial substrates to various

regimes of mechanical loading (Almekinders et al. 1993; Archambault et al. 2002; Arnoczky et al. 2002; Banes et al. 1999; Banes et al. 1995; Tsuzaki et al. 2003).

However these monolayer cell cultures may not replicate the normal *in situ* environmental conditions of tendon cells within a three dimensional, extracellular, collagenous matrix (Arnoczky et al. 2007). Since mechanotransduction signals are known to be mediated through the pericellular matrix to the nucleus via integrin based cell-matrix connections (Banes et al. 1995; Ritty et al. 2003; Sachs 1988; Wang et al. 1993; Watson 1991) it is not clear how, or even if, these complex cell-matrix interactions are maintained or recreated in cell cultures. In addition, the strain magnitudes (> 8%) and durations (> 20 hours) used to elicit an up-regulation in the expression of these inflammatory and catabolic genes may not be clinically relevant (Almekinders et al. 1993; Bhargava et al. 2004; Wang et al. 2003). Because tendon cell strain *in situ* has been shown to be appreciably less than whole tendon strain (Arnoczky et al. 2002), it is unlikely that such high levels of repetitive tendon cell strain could be reached and maintained *in vivo* without significant damage occurring within the extracellular matrix of the tendon (Woo 1982). Also, since tendons are known to exhibit non-homogeneous strain patterns in response to tensile load (Kastelic et al. 1978), it would seem impossible to precisely recreate the complex and varied patterns of strain amplitudes experienced by a population of tendon cells *in situ* by uniformly straining a large population of isolated tenocytes in monolayer. Studies have shown that in rat tail tendons even local tissue strain is nonhomogenous throughout the depth of the tendon (Arnoczky et al. 2002; Hansen et al. 2002). Thus, the *in vitro* application of high magnitudes of cyclic cellular strain to tendon cells in monolayer for excessively long durations may have little bearing

on what is actually occurring to tendon cells *in situ and* the clinical relevance of these studies must be called into question (Arnoczky et al. 2007).

Hypothesis

To better understand how the mechanotransduction response of tendon cells under tensile load affects gene expression and may contribute to the etiopathogenesis of tendinopathy, an *in situ* rat tail tendon model has been utilized in an effort to maintain the tendon cells' natural cell-matrix interactions as well as the naturally occurring strain fields that are developed in response to tensile loading (Arnoczky et al. 2004; Lavagnino et al. 2006; Lavagnino et al. 2005; Lavagnino et al. 2003). In this dissertation the mechanobiological response of tendon cells to changing loading patterns is examined and a hypothesis is forwarded that it is a mechanobiological under-stimulation resulting from altered cell-matrix interactions and not a repetitive over-stimulation of tendon cells that is the etiopathogenic stimulus for the degenerative cascade which may eventually lead to tendinopathy.

Chapter Overviews

Chapter 1 documents rat interstitial collagenase mRNA expression in an *in situ* tendon cell model in response to various cyclic loading regimes. In addition, the effect of chemically disrupting a segment of the mechanotransduction mechanism (cytoskeleton) on interstitial collagenase mRNA expression was also examined. This study suggested that removing tendons from their normal mechanical environment could significantly alter the homeostatic tension within the cytoskeletal tensegrity system and be responsible for the up-regulation of collagenase mRNA expression seen following 24 hours of stress-deprivation. In addition, the study demonstrated that interstitial collagenase mRNA

expression in tendon cells *in situ* was inhibited or even eliminated by cyclic tensile strain in a dose-dependent manner (both amplitude and frequency), presumably through a cytoskeletally based mechanotransduction pathway.

Chapter 2 investigated the potential that tendon cells may have a threshold, or set-point with regard to their mechanoresponsiveness to tensile loading. A collagen gel matrix model system was used to investigate if changes in the cytoskeletal tensional homeostasis of tendon cells was related to the control of gene expression and to determine the ability of tendon cells to re-establish their cytoskeletal tensional homeostasis in response to a changing mechanical environment. Changes in cytoskeletal tension control a reciprocal expression of anabolic and catabolic genes by tendon cells. Of particular interest in this study was the apparent ability of the tendon cells to re-establish their baseline level of internal cytoskeletal tension (as evidenced by a return to baseline gene expression) following the loss of opposing external forces offered by the collagen matrices following release.

Chapter 3 examined if an association exists between the tensile properties and the collagen fibril diameter distribution in *in vitro* stress-deprived rat tail tendons. The results of this study demonstrated that the decrease in mechanical properties observed in *in vitro* stress-deprived rat tail tendons was not correlated with the collagen fibril diameter distribution and, therefore, the collagen fibril diameter distribution does not, by itself, dictate the decrease in mechanical properties observed in *in vitro* stress-deprived rat tail tendons.

Chapter 4 examined the ability to create isolated collagen fibril damage and subsequent altered cell-matrix interactions at the damaged site using the rat tail tendon

fascicle model. This study demonstrated the creation of isolated tendon fibrillar damage within an otherwise intact tendon fascicle results in an up-regulation of collagenase mRNA expression and protein synthesis by only those tendon cells associated with the damaged fibrils. This would suggest a loss of load-transmitting function in the damaged fibril(s) and a subsequent altered cell-matrix interaction within the affected area.

Chapter 5 builds on the experimental study of Chapter 1 with the creation of a multiscale computational tendon model composed of both matrix and fluid phases to examine how global tendon loading may affect fluid-flow-induced shear stresses and membrane strains at the cellular level. The model analysis, combined with additional experimental results, demonstrated that both strain rate and strain amplitude are able to independently alter rat interstitial collagenase gene expression through increases in fluid-flow-induced shear stress and matrix-induced cell deformation respectively.

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CHAPTER 1

Effect of Amplitude and Frequency of Cyclic Tensile Strain on the Inhibition of MMP-13 mRNA Expression in Tendon Cells: an *in vitro* study

Michael Lavagnino
Steven P. Arnoczky
Tao Tian
Zachary Vaupel

From the Laboratory for Comparative Orthopaedic Research
College of Veterinary Medicine, Michigan State University,
East Lansing, Michigan 48824, USA

Lavagnino, M, Arnoczky, SP, Tian, T and Vaupel, Z (2003) Effect of amplitude and frequency of cyclic tensile strain on the inhibition of MMP-1 mRNA expression in tendon cells: an in vitro study. Connect Tissue Res 44:181-187.

ABSTRACT

To determine the effect of cyclic strain amplitude and frequency on MMP-13 (interstitial collagenase) expression in tendon cells, rat tail tendons (RTT) were immobilized or cyclically displaced to various amplitudes (1, 3, or 6% strain at 0.017 Hz) or frequencies (1% strain at 0.017, 0.17, or 1.0 Hz) for 24 hr. Stress-deprivation for 24 hr resulted in a marked upregulation in MMP-13 expression. Cyclic tensile loading at 0.017 Hz was found to significantly inhibit, but not completely eliminate, MMP-13 expression at 1% strain. MMP-13 expression was completely eliminated at 3 and 6% strain. Increasing the frequency of application of the 1% strain to 0.17 or 1.0 Hz completely eliminated MMP-13 expression. Disruption of the actin cytoskeleton with cytochalasin D abolished all inhibitory effects of cyclic strain on MMP-13 expression. The results of our study demonstrate that MMP-13 expression in tendon cells can be modulated by varying amplitudes and frequencies of cyclic tensile strain, presumably through a cytoskeletally based mechanotransduction pathway.

INTRODUCTION

Stress deprivation has been shown to have deleterious effects on the structural and functional properties of ligaments and tendons (Amiel et al. 1982; Boorman et al. 1998; Gamble et al. 1984; Goomer et al. 1999; Hannafin et al. 1995; Loitz et al. 1989; Majima et al. 2000; Majima et al. 1994; Noyes 1977). These effects appear to be cell mediated (Hannafin et al. 1995) and are thought to involve interstitial collagenase based alterations of the extracellular matrix (Goomer et al. 1999; Loitz et al. 1989). Previous *in vitro* studies have implicated a stress-suppressible effect of tensile stress on interstitial collagenase expression in ligaments (Hannafin et al. 1995; Loitz et al. 1989; Majima et al. 2000). A recent study from our lab has demonstrated that *in situ* stress-deprivation of rat tail tendon cells resulted in an immediate up-regulation of rat interstitial collagenase (MMP-13) mRNA expression (Arnoczky et al. 2004). Application of static tensile loading produced a dose-dependent inhibition of MMP-13 mRNA expression through a cytoskeletally based mechanotransduction mechanism (Arnoczky et al. 2004). However, this inhibition was incomplete at the physiological stresses examined (Arnoczky et al. 2004).

In bone, cyclic loading has been shown to be more effective than static loading in inhibiting catabolic activity and stimulating anabolic processes of cells (Burger and Klein-Nulen 1999; Burger and Klein-Nulend 1999; Jacobs et al. 1998). Cyclic loading of bone produces oscillatory fluid flow within the lacunar/canalicular network (Burger and Klein-Nulend 1999). This fluid flow, and the resulting shear stress, has been shown to be an important physical signal that influences bone cell metabolism and bone adaptations to mechanical loading (Burger and Klein-Nulen 1999; Burger and Klein-Nulend 1999;

Hsieh and Turner 2001; Hung et al. 1995; Jacobs et al. 1998; Owan et al. 1997; You et al. 2000). Indeed, *in vitro* studies have shown that, at low levels (<0.2%) of tissue strain, fluid flow is a more potent stimulator of bone cells than is matrix deformation itself (You et al. 2000). These findings have led to the successful application of low amplitude, high frequency mechanical stimulation in bone to inhibit catabolism (Rubin et al. 2001).

Cyclic loading of tendons has been shown to produce interstitial fluid flow (Butler et al. 1997; Chen et al. 1998; Hannafin and Arnoczky 1994; Lanir et al. 1988), which in turn, has been linked to gene expression through a mechanotransduction process involving a cytoskeletal tensegrity system (Archambault et al. 2002). It is possible that lower amplitudes and increased frequency of repetitive cyclic tensile loading may have a more profound effect on maintaining tendon health than higher amplitudes of low frequency or static loading.

Therefore, the purpose of this study was to examine the effect of various amplitudes and frequencies of cyclic tensile strain on the regulation of MMP-13 expression in rat tail tendon cells. It was our hypothesis that cyclic tensile strain will inhibit MMP-13 expression in tendon cells in a dose dependent (amplitude and frequency) manner through a cytoskeletally based mechanotransduction pathway.

MATERIALS AND METHODS

Drugs and Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Ascorbate, gentamicin, and penicillin-streptomycin-fungizone solution were obtained from Gibco (Grand Island, NY, USA). Cytochalasin D, a fungal product that

depolymerizes actin filaments of the cytoskeleton, was obtained from SIGMA (St. Louis, MO, USA).

Tendon Culture

Following institutional animal care and use approval, tendons were obtained from the tails of adult Sprague Dawley rats. The tendons were removed immediately after euthanasia and maintained in DMEM media supplemented with 10% FBS, antibiotic/antimycotic solution, and Ascorbate at 37°C and 10% CO_2 for the duration of the experiments.

Experimental Groups

Tendons were divided into groups for three experiments. Each experiment had a zero time control group of fresh rat tail tendons. The first experiment varied cyclic strain amplitude as follows: *Group 1*: stress-deprived (no strain) for 24 hours; *Group 2*: 1% cyclic strain at 0.017Hz for 24 hours; *Group 3*: 3% cyclic strain at 0.017Hz for 24 hours; *Group 4*: 6% cyclic strain at 0.017Hz for 24 hours. The second experiment varied cyclic strain frequency as follows: *Group 1*: stress-deprived (no strain) for 24 hours; *Group 2*: 1% cyclic strain at 0.017Hz for 24 hours; *Group 3*: 1% cyclic strain at 0.17Hz for 24 hours; *Group 4*: 1% cyclic strain at 1.0Hz for 24 hours. The third experiment evaluated the effect of cytochalasin D (SIGMA) as follows: *Group 1*: stress-deprived (no strain) for 24 hours; *Group 2*: 6% cyclic strain at 0.017Hz for 24 hours; and *Group 3*: 6% cyclic strain at 0.017Hz with 10μM cytochalasin D for 24 hours. There were twenty tendons per group and each experiment was repeated three times.

Testing Setup

Stress-deprived tendons were kept in a 60mL dish in complete media and conditions as described above for 24 hours. A sawtooth-shaped waveform of cyclic strain was applied to tendons using a custom made, computer-controlled, stepper motor-driven device (Figure 1.1). The grip-to-grip length was set to 40mm using digital calipers

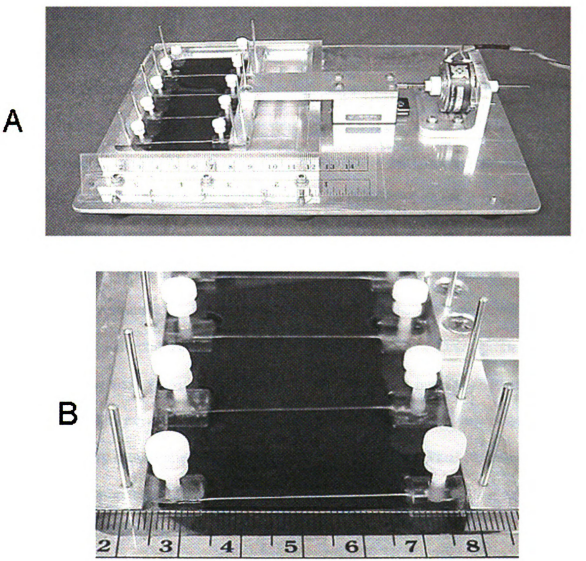


Figure 1.1 A: Photograph of the computer-controlled, stepper motor driven, cyclic loading system. The system permits five tendons to be loaded simultaneously while in media. **B**: Close up view of the testing system showing rat tail tendons in place.

The grip-to-grip length was set to 40mm using digital calipers (Mitutoyo, Tokyo, Japan). Tendons were placed in the device until all visible slack was removed to approximate 0% strain. Tendons were then clamped in the grips to prevent slipping before undergoing 1, 3, or 6% cyclic strain with a step size of 25μm and a rate of 0.017, 0.17, or 1Hz. At 1% strain, a frequency of 0.017Hz corresponds to a strain rate of 0.033% strain/second and a total of 1440 load events over the 24-hour testing period. At strains of 3 and 6% this strain rate increases to 0.1 and 0.2% strain/second, respectively. Increasing the frequency to 0.17Hz results in a strain rate of 0.33% strain/second and a total of 14,400 load events, whereas a frequency of 1.0Hz corresponds to a strain rate of 2% strain/second and results in a total of 86,400 load events during the 24-hour test period.

Tendon Analysis

At the end of the experimental period, the unclamped tendon segment (~40 mm) of the cyclically strained tendons and the entire length of the stress-deprived tendons were collected and total cellular RNA was hybridized with a DNA probe for rat interstitial collagenase (MMP-13) generated in our lab and a human GAPDH cDNA control probe. The exposed films were scanned and MMP-13 expression was quantified by optical density measurements and standardized as a ratio of GAPDH expression. The effect of strain amplitude, strain frequency, and cytochalasin D on MMP-13 expression was evaluated using an ANOVA and Tukey's post-hoc test. Significance was set at $p<0.05$.

Construction of MMP-13 DNA Plasmid

To prepare the probe, MMP-13 DNA (GeneBank M60616) was amplified by polymerase chain reaction (PCR) using rat genomic DNA template. The primers used

were 5'-GCC CAT ACA GTT TGA ATA CAG TAT CTG-3' and 5'-CCA GTT TAA TAA ACA CCA TCT CTT GA-3'. PCR product (1167-bp) was subjected to electrophoresis on 1% agarose gel and recovered using Qiaex II Kit (QIAGEN Inc., Valencia, CA, USA) and then cloned into pCR®2.1-TOPO® plasmid using TOPO TA Cloning kit (Invitrogen Living Science, Carlsbad, CA, USA). The plasmid was transformed into competent *E.coli* with selection for kanamycin and ampicillin resistance. The construction was confirmed by restriction enzyme digestion (EcoR I, EcoR V, and Hind III) and polymerase chain reaction.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was isolated from rat tail tendons by the acid guanidine thiocyanate-phenol-chloroform procedure (totally RNA kit, Ambion Inc., Austin, TX, USA). The RNA samples were subjected to electrophoresis on 1.2% agarose gels containing 0.66M formaldehyde and MOPS, then transferred to a nylon membrane (Pierce Corp, Rockford, IL, USA) for 1 hour in TAE at 300mA. Following transfer, the membrane was air-dried and UV cross-linked at 10 Joules/cm^2.

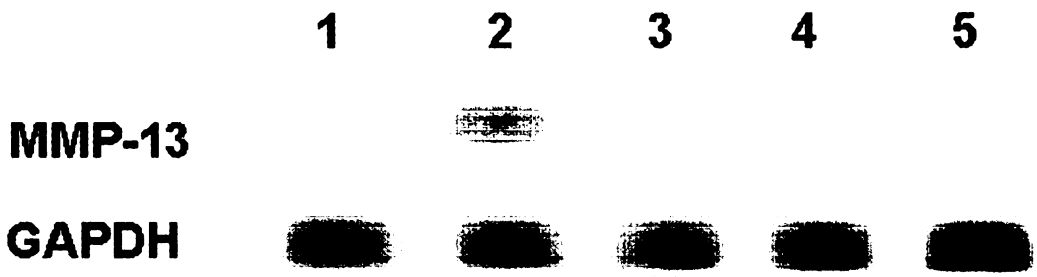
The MMP-13 probe and human GAPDH cDNA control probe (Clontech Laboratories, Inc., Palo Alto, CA, USA) were labeled with biotin using the North2South Direct HRP Labeling and Detection Kit (Pierce Corp). The RNA blots were hybridized with labeled probes (10ng/ml hybridization solution) at 55°C for 1 hour. The membrane was then washed with 40 ml (~0.5 mL per cm^2) 2x SSC/0.1% SDS at 55°C (3 x 5 minutes) and washed with 40 ml (~0.5 mL per cm^2) of 2x SSC at room temperature.

Following wash, the membrane was incubated with chemiluminescent working solution for 5 minutes and exposed to films for 5-10 minutes. The films were scanned

with a laser film digitizer (Lumiscan 75, Lumisys Inc., Sunnyvale, CA, USA) and MMP-13 mRNA expression was quantitated by optical density measurements using Scion Image (Scion Corporation, Frederick, MD, USA).

RESULTS

In all experiments, stress-deprivation for 24 hours resulted in a significant ($p<0.05$) up-regulation of MMP-13 expression in tendon cells compared to fresh tendons (Figures 1.2-1.4).



Lane 1	fresh tendon (0 time)
Lane 2	no strain -24 hrs
Lane 3	1% cyclic strain @0.017Hz -24hrs
Lane 4	3% cyclic strain @0.017Hz -24hrs
Lane 5	6% cyclic strain @0.017Hz -24hrs

Figure 1.2 Representative Northern blot gel from the amplitude experiment illustrating the relative expression of MMP-13 mRNA expression in fresh control tendons (lane 1); immobile for 24 hours (lane 2); 1% cyclic strain at 0.017Hz for 24 hours (lane 3); 3% cyclic strain at 0.017Hz for 24 hours (lane 4); 6% cyclic strain at 0.017Hz for 24 hours (lane 5). GAPDH was used as an internal control. Experiments were performed three times and a representative result is shown.

Amplitude

A low cyclic strain amplitude of 1% at 0.017Hz resulted in a significant ($p<0.05$), but incomplete, inhibition of MMP-13 expression. Increasing the cyclic amplitude to 3 or 6% strain at 0.017Hz completely eliminated MMP-13 expression (Figure 1.2).

Frequency

A low cyclic strain frequency of 0.017Hz at 1% strain again resulted in a significant ($p<0.05$), but incomplete, inhibition of MMP-13 expression. Increasing the cyclic frequency to 0.17 or 1.0Hz completely eliminated MMP-13 expression (Figure 1.3).

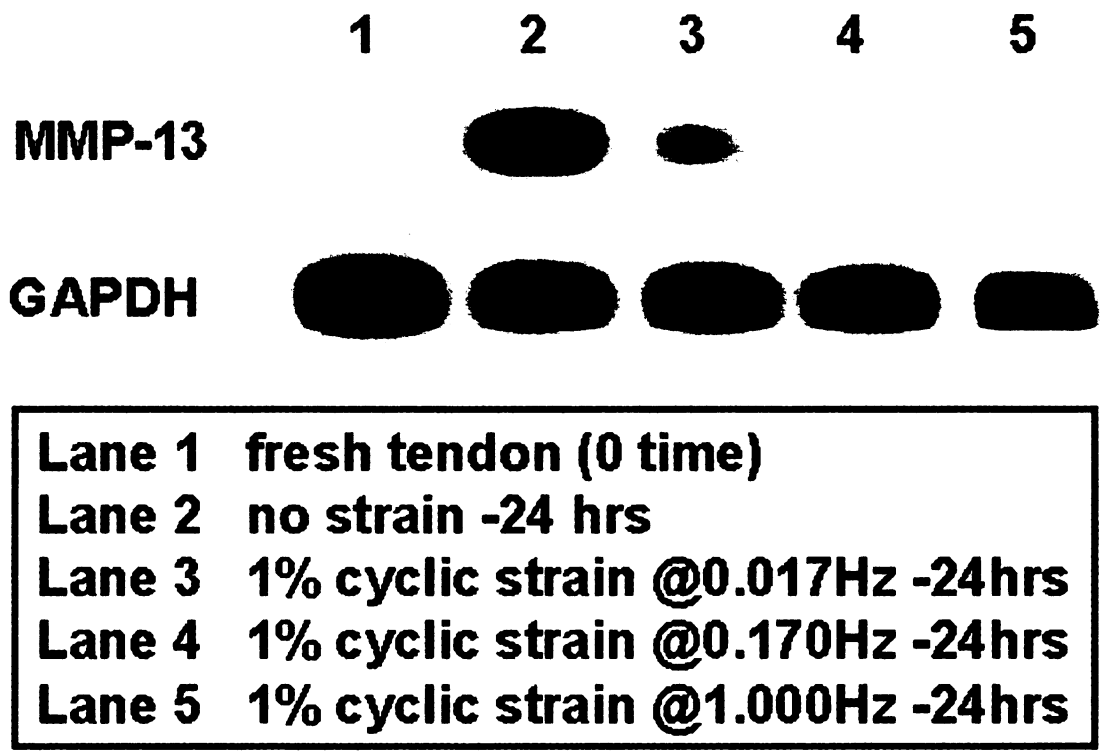
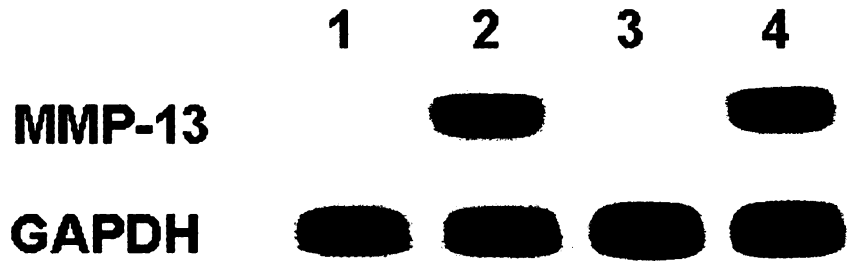


Figure 1.3 Representative Northern blot gel from the frequency experiment illustrating the relative expression of MMP-13 mRNA expression in fresh control tendons (lane 1); immobile for 24 hours (lane 2); 1% cyclic strain at 0.017Hz for 24 hours (lane 3); 1% cyclic strain at 0.17Hz for 24 hours (lane 4); 1% cyclic strain at 1.0Hz for 24 hours (lane 5). GAPDH was used as an internal control. Experiments were performed three times and a representative result is shown.

Cytochalasin D

Disruption of the actin cytoskeleton in rat tail tendon cells abrogated the inhibitory effect of cyclic loading on MMP-13 expression. There was no significant ($p=0.56$) difference in MMP-13 expression between tendons stress-deprived for 24 hours and tendons exposed to 6% cyclic strain at 0.017Hz and 10μM cytochalasin D for 24 hours (Figure 1.4).



Lane 1 **fresh tendon (0 time)**
Lane 2 **no strain -24 hrs**
Lane 3 **6% cyclic strain @0.017Hz -24hrs**
Lane 4 **6% cyclic strain @0.017Hz + cytochalasin -24hrs**

Figure 1.4 Representative Northern blot gel from the cytochalasin D experiment illustrating the relative expression of MMP-13 mRNA expression in fresh control tendons (lane 1); immobile for 24 hours (lane 2); 6% cyclic strain at 0.017Hz for 24 hours (lane 3); 6% cyclic strain at 0.017Hz for 24 hours with 10 μM of cytochalasin D for 24 hours (lane 4). GAPDH was used as an internal control. Experiments were performed three times and a representative result is shown.

DISCUSSION

Application of load to bone (Brighton et al. 1991; Burger and Klein-Nulen 1999; Hsieh and Turner 2001; Huiskes et al. 2000; Neidlinger-Wilke et al. 1994; Rubin et al. 2001), ligament (Hannafin et al. 2006; Hsieh et al. 2000), and tendon (Arnoczky et al. 2004; Arnoczky et al. 2002; Banes et al. 1995; Hannafin et al. 1995) has been implicated in the maintenance of tissue homeostasis. This is thought to occur through the transfer of tissue strain to the cell cytoskeleton that, in turn, initiates a mechanotransduction signaling response (Banes et al. 1995; Ingber et al. 1995; Sachs 1988). Transmission of tendon strain to the extracellular matrix and cells has not been completely determined, although substrate (extracellular matrix) strain and fluid flow are potential mechanisms (Archambault et al. 2002; Takai et al. 1991).

Previous *in vitro* studies in ligaments and tendons have shown that tensile loading produces substrate (extracellular matrix) strain that, in turn, alters cell shape (Arnoczky et al. 2002; Matyas et al. 1994). Changes in cell shape and the resulting alterations in the actin cytoskeleton are key components in the mechanotransduction response(s) of cells (Banes et al. 1995; Ingber et al. 1995; Sachs 1988; Wang et al. 1993; Watson 1991). A recent study has shown that when static tensile load is applied to rat tail tendons, rat MMP-1 (MMP-13) mRNA expression in tendon cells is inhibited in a dose dependent manner (Arnoczky et al. 2004). This amplitude-dependent inhibition of MMP-13 expression appears to correlate with the progressive loss of collagen crimp (from the surface to the center of the rat tail tendon) and the increase in fiber recruitment reported in tendon fascicles with increasing stresses (Hansen et al. 2002). Thus, sequential increases in substrate strain likely result in an increasing number of cells being deformed

(Arnoczky et al. 2002). However, because MMP-13 mRNA expression was only inhibited and not totally eliminated with what would appear to be physiologic levels of static stress, substrate deformation may not be the sole factor, or even the most important factor involved in tendon cell signaling and subsequent gene expression with tensile load.

Fluid flow and the resultant shear stress are thought to be other important mechanisms for the transmission of tissue strain to cells (You et al. 2000). Shear strain and an up-regulation in gene expression result when musculoskeletal cells in monolayer are exposed to fluid flow (Archambault et al. 2002; Burger and Klein-Nulen 1999; Hung et al. 1997; Hung et al. 1995; Jacobs et al. 1998; Owan et al. 1997; Xu et al. 2000; You et al. 2000). With bone cells, fluid flow is thought to play a significantly greater role than substrate deformation in activating gene expression (Owan et al. 1997; You et al. 2000). While cyclic strain-induced fluid flow in bone occurs through a patent and well-developed canalicular network, the flow of interstitial fluid in response to cyclic loading is less defined in tendons (Archambault et al. 2002; Hannafin and Arnoczky 1994). Researchers modeling the interstitial fluid flow in tendons have proposed that tensile loading, and the resulting fluid flow, exert a mechanotranduction effect on the tendon cells through shear strain and pressure; however, the exact levels of fluid-induced shear stress have not been identified (Butler et al. 1997; Chen et al. 1998). More research is necessary to determine the precise role of cyclic tensile loading, fluid flow, and shear stress in the mechanotransduction response of tendon cells *in situ*.

In the current study, increasing the cyclic strain frequency totally eliminated MMP-13 mRNA expression at low amplitude strain levels. Similar effects have been reported in other cell types (Yang et al. 1998). Low amplitude (1%) cyclic straining at

1Hz is effective in suppressing interstitial collagenase production in human vascular smooth muscle cells (Yang et al. 1998). Whereas the enhanced response of the cells in the current study may be directly related to the increase in frequency of tensile loading, it also may be a result of the increase in strain rate and/or the increase in the total number of load events associated with increases in loading frequency. Frequency, strain rate, and/or number of loading cycles potentially could have a threshold effect on MMP-13 mRNA expression.

While the role of strain rate and number of loading cycles on MMP-13 mRNA expression remains to be elucidated, an *in vivo* study examining the role of cyclic loading in tendon healing suggests increased loading frequency and not an increase in total load events is responsible for improved mechanical properties in healing tissues (Takai et al. 1991). In that study, loading frequencies (0.017 and 0.2Hz) similar to those used in the current study were compared, but the number of loading events for each frequency remained constant (Takai et al. 1991). The authors reported a significant improvement in the mechanical properties of tendons loaded at the higher frequency (Takai et al. 1991). However, the exact mechanism for this improvement (i.e., inhibition of catabolism or stimulation of anabolism) was not determined.

The loading frequencies and amplitudes utilized in this study were commensurate with those used in previous *in vivo* (Majima et al. 2000; Takai et al. 1991) and *in vitro* (Hannafin et al. 1995; Yang et al. 1998) studies and reflect accepted physiologic levels for normal tendon activity (Viidik 1990). Since tendons are known to exhibit nonhomeogeneous strain patterns in response to tensile load (Kastelic et al. 1978), it is impossible to determine precise amplitudes of strain experienced by the cells based on

overall tendon strain. Studies have shown that in rat tail tendons even local tissue strain is nonhomogenous throughout the depth of the tendon (Arnoczky et al. 2002; Hansen et al. 2002). The overall tissue strains used in this study are within the normal functional range of tendons and the response of interstitial collagenase mRNA expression to tensile load reported in the current study is similar to those reported for an *in vivo* ligament and tendon study (Majima et al. 2000).

As stated, the strain-induced mechanotransduction response is thought to be mediated through the cytoskeleton (You et al. 2000). Changes in cell shape, specifically the loss of actin stress fiber organization, has been strongly correlated with collagenase gene expression (Aggeler et al. 1984; Arnoczky et al. 2004). Procollagenase expression in rabbit synovial fibroblasts was induced by treatments that modified cellular actin (Aggeler et al. 1984). Collagenase synthesis was upregulated within six hours of cytoskeletal alteration (Aggeler et al. 1984) and would suggest that a change in the state of actin assembly has a rapid influence on the kinetics of collagenase mRNA expression. In the current study, treatment cytochalasin D, a fungal product that depolymerizes actin filaments, completely abrogated the cyclic strain-induced inhibition of MMP-13 mRNA expression. These results match similar findings with static load (Arnoczky et al. 2004) and further support the role of a cytoskeletally based mechanosensory tensegrity system in the control of MMP-13 mRNA expression in tendon cells.

A limitation of this study is that only MMP-13 mRNA expression and not protein synthesis was examined. Since gene expression may not correlate directly with synthesis of the active enzyme, the association between stress-deprivation induced MMP-13 expression and extracellular matrix degradation was not examined in this study. While

stress-deprivation and cyclic load may have an effect on the regulation of other MMPs, previous *in vivo* studies have suggested that interstitial collagenase appears to be the major matrix metalloproteinase associated with immobilization-induced alterations of tendons and ligaments (Goomer et al. 1999; Majima et al. 2000). Finally, the current study only examined the ability of cyclic load to inhibit the upregulation of the MMP-13 gene. Future studies are needed to determine the effects of cyclic loading on downregulating MMP-13 mRNA expression after its expression has been upregulated.

The results of our study demonstrate that MMP-13 mRNA expression in tendon cells *in situ* can be modulated by cyclic tensile strain in a dose-dependent manner (both amplitude and frequency), presumably through a cytoskeletally based mechanotransduction pathway. Understanding the role of exercise on gene expression may help determine optimal exercise protocols for both injured and healthy tissues through the controlled application of load and frequency. It is possible that lower amplitudes and increased frequency of repetitive cyclic tensile loading may have a more profound effect on maintaining tendon health than higher amplitudes of low frequency or static loading. This could lead to advances in overuse injury prevention and optimal rehabilitation protocols following tendon injury and repair.

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CHAPTER 2

In vitro Alterations in Cytoskeletal Tensional Homeostasis Control Gene Expression in Tendon Cells

Michael Lavagnino
Steven P. Arnoczky

From the Laboratory for Comparative Orthopaedic Research
College of Veterinary Medicine, Michigan State University,
East Lansing, Michigan 48824, USA

Lavagnino, M and Arnoczky, SP (2005) In vitro alterations in cytoskeletal tensional homeostasis control gene expression in tendon cells. J Orthop Res 23:1211-1218.

ABSTRACT

An in vitro collagen gel system was used to determine the effect of alterations in cytoskeletal tensional homeostasis on gene expression in tendon cells. Collagen gel matrices, seeded with rat tail tendon cells, underwent cytochalasin D and gel contraction treatments designed to alter the internal cytoskeletal homeostasis of the cells. Gels were examined for cytoskeletal organization using a rhodamine phalloidin stain for actin. The effect of altered cytoskeletal organization on mRNA expression of a catabolic (interstitial collagenase) and anabolic (alpha1(I) collagen) gene was examined using northern blot analysis. Tendon cells in adhered gels demonstrated a highly organized cytoskeleton and showed evidence of alpha1(I) collagen mRNA expression but no evidence of collagenase mRNA expression. Treatment of the attached gel with cytochalasin D disrupted the cytoskeletal organization and resulted in the up-regulation of collagenase mRNA and the inhibition of alpha1(I) collagen mRNA expression. Release of the gels resulted in a cell mediated gel contraction, an immediate loss of cytoskeletal organization, and an mRNA expression pattern similar to that seen with cytochalasin D treatment. Isometric contraction of the gel on itself or around a 3-point traction device resulted in an mRNA expression pattern similar to the adhered gel. Gene expression in the contracted gels could be reversed through chemical cytoskeletal disruption or removal of the traction device which permitted further gel contraction. The results of the study suggest that tendon cells can establish an internal cytoskeletal tension through interactions with their local extracellular environment. Alterations in this tension appear to control the expression of both catabolic and anabolic genes in a reciprocal manner.

INTRODUCTION

Mechanoresponsiveness is a fundamental feature of all living tissues (Brown et al. 1998; Ingber 1991; Ingber 1997) and tendons are no exception (Banes et al. 1995). Experiments with cultured tendon cells in monolayer confirm that mechanical stresses can regulate a wide variety of cellular processes including signal transduction, gene expression, and proliferation (Almekinders et al. 1993; Archambault et al. 2002; Arnoczky et al. 2002; Banes et al. 1999; Banes et al. 1994; Banes et al. 1995; Tsuzaki et al. 2003; Tsuzaki et al. 2003). However, the precise level of mechanical load required to initiate (or inhibit) specific cell processes has not been rigorously investigated.

Recent *in vitro* studies have shown that stress deprivation of tendon cells *in situ* results in an immediate up-regulation of rat interstitial collagenase via a cytoskeletally based mechanotransduction mechanism (Arnoczky et al. 2004; Lavagnino et al. 2003). Conversely, application of a tensile load has been shown to inhibit mRNA expression of interstitial collagenase in a dose dependent manner; presumably, through the same cytoskeletally based mechanism (Arnoczky et al. 2004). These results suggest that tendon cells may have a threshold, or set-point, with regard to their mechanoresponsiveness to tensile loading.

Frost first proposed the concept of the mechanostat set-point to explain the mechanoresponsiveness of bone cells in controlling bone mass (Frost 1987). He theorized that bone cells are programmed to sense a certain level of strain induced signals. If the signal was below the set-point the cell would activate catabolic mechanisms that decrease bone mass (Frost 1987). Conversely, if the strain signal exceeded the set-point, anabolic mechanisms would be activated to increase bone mass

(Frost 1987). While this concept provides an explanation of how bone mass adapts to gross overloading and underloading, a recent study has suggested that bone cells can also autoregulate their sensitivity to a strain-induced signal by altering their local microenvironment (Rubin et al. 1999). It was theorized that in response to subtle changes in mechanical stress bone cells could actively tune their microenvironment to maintain their idealized strain environment (Rubin et al. 1999).

A similar response has been observed in fibroblasts seeded into collagen gels. These cells have been shown to generate a homeostatic contractile force within their extracellular collagenous matrix (Brown et al. 1998). This is achieved through the creation of tension within the internal cytoskeleton via an actomysin filament sliding mechanism (Chen and Ingber 1999; Dickinson et al. 1994; Skalak et al. 1994). The cells reciprocally increased or decreased their endogenous contractions against changes to opposing external loads (Brown et al. 1998). This response allowed the fibroblasts to respond to perceived changes in mechanical loading in a way that maintained tensional homeostasis between the cell and its surrounding extracellular matrix (Brown et al. 1998). It is probable that cytoskeletal tensional homeostasis is the mechanism by which tendon cells establish and attempt to maintain their mechanostat set-point.

The purpose of this study was to determine if changes in the cytoskeletal tensional homeostasis of tendon cells are related to the control of gene expression and to determine the ability of tendon cells to re-establish their cytoskeletal tensional homeostasis in response to a changing mechanical environment. Our hypotheses were that tendon cells can generate an internal tensional homeostasis which calibrates the cell with respect to gene expression (rat interstitial collagenase and α1(I) collagen) and that alterations in this

internal stress cause a reciprocal change in the expression of catabolic (interstitial collagenase) and anabolic (α1(I) collagen) genes.

MATERIALS AND METHODS

Cell Culture

Rat tail tendon cells were harvested via primary explant cultures from adult Sprague-Dawley rats euthanized for another unrelated study. The cells were expanded to passage 3 in 75 cm^2 tissue culture flasks in Dulbecco's modified Eagle medium, 10% fetal bovine serum, Ascorbate (150 mg/ml), 0.01 mg/ml gentamicin, and 1% antibiotic/antimycotic solution (Gibco, Grand Island, NY, USA) at 37°C in a 10% CO_2 atmosphere.

Collagen Gel

Collagen gels made of 2.4 mg/ml type I bovine collagen (Vitrogen, Cohesion Technologies, Palo Alto, CA, USA) were seeded with rat tail tendon cells at a concentration of 400,000 cells/mL. Two milliliter gels were created in individual 60 mm culture dishes and allowed to adhere to the culture dishes for at least 24 hours before treatment. The gels were incubated in complete media composed of Dulbecco's modified Eagle medium, 10% fetal bovine serum, Ascorbate, and penicillin-streptomycin-fungizone (Gibco, Grand Island, NY, USA) at 37°C and 10% CO_2. The gels were divided into groups to examine the effects of either chemical or physical treatments on cytoskeletal organization and subsequent gene expression. At the end of the study, gels were frozen at –80°C until processed for Northern blot analysis. Each group consisted of five gels and was repeated three times. Additional gels were used to examine cytoskeletal

organization in attached, cytochalasin D treated, and released gels using a rhodamine phalloidin stain.

Contraction and Chemical Alteration of the Cytoskeletal Tension

Seeded collagen gels were allowed to attach to the culture dishes for 24 hours and then treated as follows: *Group 1:* left attached to the culture dishes for an additional 24 hours; *Group 2:* attached to the culture dishes for an additional 24 hours while exposed to 10 μM cytochalasin D (SIGMA, St. Louis, MO, USA) to disrupt the cellular cytoskeleton; *Group 3:* released from the culture dishes and allowed to contract for 24 hours; *Group 4:* released and allowed to contract for 10 days; *Group 5:* released from the culture dishes and allowed to contract for 10 days, and then exposed to 10μM cytochalasin D to disrupt the cellular cytoskeleton for an additional 24 hours; *Group 6:* released from the culture dishes and allowed to contract for 14 days.

Contraction and Physical Alteration of the Cytoskeletal Tension

To study response of tendon cells to changing extracellular environments an external 3-point traction device was inserted into the gels immediately following release of the gels from the bottom of the culture dishes, such that the gels contracted around the three stainless steel pins (Figure 2.1). The pins in the device were arranged in an equilateral triangular fashion with 12 mm between each pin. This experiment consisted of allowing seeded collagen gels to attach to the culture dishes for 24 hours and then treating them as follows: *Group 1:* left attached to the culture dishes for an additional 24 hours*; Group 2:* released from the culture dishes and allowed to contract for 24 hours around the 3-point traction pins; *Group 3:* released from the culture dishes and allowed to contract for 10 days around 3-point traction pins; *Group 4:* released from the culture

dishes, allowed to contract for 10 days around 3-point traction pins, and then removal of the pins from the gel allowing for an additional 24 hours of contraction.

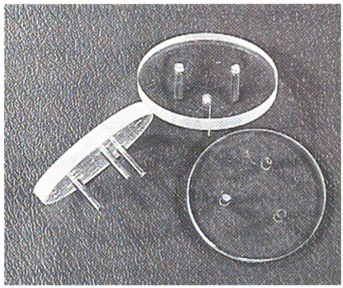

Figure 2.1 Photograph showing the 3-point traction devices.

Construction of DNA plasmids to rat interstitial collagenase and rat α1(I) collagen

To prepare the collagenase probe, rat interstitial collagenase DNA (GeneBank M60616) was amplified by polymerase chain reaction using rat genomic DNA template. The primers used were 5'-GCC CAT ACA GTT TGA ATA CAG TAT CTG-3' and 5'-CCA GTT TAA TAA ACA CCA TCT CTT GA-3'. PCR product (1167-bp) was subjected to electrophoresis on 1% agarose gel and recovered using Qiaex II Kit (QIAGEN Inc., Valencia, CA, USA) and then cloned into pCR®2.1-TOPO® plasmid using TOPO TA Cloning kit (Invitrogen Living Science, Carlsbad, CA, USA). The plasmid was transformed into competent E. coli with selection for kanamycin and ampicillin resistance. The construction was confirmed by restriction enzyme digestion (EcoR I, EcoR V, and Hind III) and polymerase chain reaction.

To prepare the collagen probe, rat α1(I) collagen DNA (GeneBank M60616) was amplified by reverse transcription-polymerase chain reaction with total RNA purified from cultured rat tail tendon fibroblasts. The oligonucleotide primers used were 5'-GTC CAT TCC GAA TTC CTG GTC-3' and 5'GTC GCA CTG GCG ATA GTG G-3'. PCR product (866-bp) was subjected to electrophoresis on 1% agarose gel and recovered using a Qiaex II Kit (QIAGEN Inc., Valencia, CA, USA), and then cloned into pZErO™-2 plasmid using a Zero Background™/Kan Cloning kit (Invitrogen Living Science, Carlsbad, CA, USA). The plasmid was transformed into competent E. coli with selection for kanamycin resistance. The construction was confirmed by restriction enzyme digestion (ECOR I and Hind III) and polymerase chain reaction.

RNA Extraction and Northern Blot Analysis

A Northern blot analysis was performed to assay for rat interstitial collagenase mRNA expression and α1(I) collagen mRNA expression. The gels within each experimental group were combined and total cellular RNA isolated by the acid guanidine thiocyanate-phenol-chloroform procedure (totally RNA kit, Ambion Inc., Austin, TX, USA). The RNA samples were subjected to electrophoresis on 1.2% agarose gels containing 0.66M formaldehyde and MOPS, then transferred to a nylon membrane (Pierce Corp, Rockford, IL, USA) for 1 hour in TAE at 300 mA. Following transfer, the membrane was air-dried and UV cross-linked at 10 J/cm^2.

The rat interstitial collagenase probe and α1(I) collagen probe, as well as a human GAPDH cDNA control probe (Clontech Laboratories, Inc., Palo Alto, CA, USA) were labeled with biotin using the North2South Direct HRP Labeling and Detection Kit (Pierce Corp, Rockford, IL, USA). The RNA blots were hybridized with labeled probes (10

ng/ml hybridization solution) at 55°C for 1 hour. The membrane was then washed with 40 mL (~ 0.5 mL/cm^2) 2x SSC/0.1% SDS at 55°C (3x 5 minutes) and washed with 40 mL (~ 0.5 mL/cm^2) of 2x SSC at room temperature. Following the wash procedure, the membrane was incubated with chemiluminescent working solution for 5 minutes and exposed to films for 5-10 minutes.

Cytoskeletal Organization

To evaluate alterations in cytoskeletal organization following chemical or physical manipulations, additional gels were prepared for actin staining and confocal laser microscopy. Gels were fixed in 10% phosphate buffered formalin and stained with rhodamine phalloidin (5 units/mL) (Molecular Probes, Eugene, Oregon, USA) to examine the actin filament structure of the cells after the following treatments: a gel adhered to a culture dish for 48 hours, a gel adhered to a culture dish for 24 hours and then exposed to 10μm cytochalasin D for 1 hour, and a gel adhered to a culture dish for 24 hours and then released and allowed to contract for 5 minutes. The gels were wet mounted on a glass slide for viewing with a Zeiss Pascal Laser Scanning Confocal microscope (Carl Zeiss, Thornwood, NY, USA). Observations were made using a 40x oil immersion objective without a coverslip. Fluorescent images were obtained using a HeNe 543 nm laser with a long pass 560 nm emission filter. Cell shape and actin stress fiber organization were observed in cells throughout the gel. After localizing a representative cell in each gel, a 2x zoom was used to obtain an image.

RESULTS

Cytoskeletal Organization

In gels adhered to the culture dish for 48 hours, the tendon cells appeared elongated and their cytoskeletons contained well-organized actin stress fibers (Figure 2.2A). The addition of cytochalasin D to the adhered gels or the physical release of the gels from the culture dish resulted in an immediate loss of this actin stress fiber organization (Figure 2.2B and C).

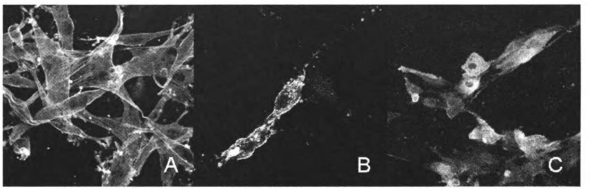

Figure 2.2 Representative rhodamine-phalloidin stained cell images under confocal microscopy (40x) of A) elongated cells in adhered gels at 48 hours containing well-organized actin stress fibers, B) the addition of cytochalasin D to the adhered gels or C) the physical release of the gels from the culture dish resulted in an immediate loss of actin stress fiber organization.

Contraction and Chemical Alteration of the Cytoskeletal Tension

Upon release from their attachment to their individual culture dishes, the tendon cell seeded collagen gels were contracted by the tendon cells (Figure 2.3). The contraction continued over the next two weeks condensing the gel into a dense, circular bead at 14 days (Figure 2.3).

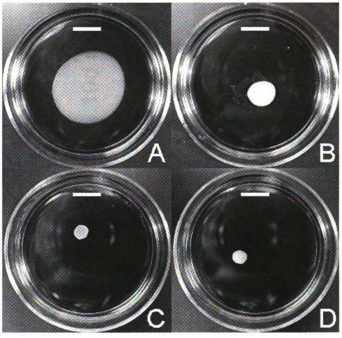

Figure 2.3 Photograph showing a representative gel after 48 hours of attachment to its culture dish **(A)** and the contraction of the gel following release after 24 hrs **(B)**, 10 days **(C)**, and 14 days **(D)**. (Scale bar = 10 mm).

In each of the groups examined, it was apparent that there was a reciprocal relationship between rat interstitial collagenase and α1(I) collagen mRNA expression such that when one was expressed, the other was not (Figure 2.4). Tendon cells in adhered gels demonstrated expression of α1(I) collagen mRNA but no measurable expression of rat interstitial collagenase mRNA (Lane 1). Disruption of cytoskeletal stress fiber organization from exposure to cytochalasin D (lane 2) or gel release and

contraction for 24 hours (lane 3) produced an up-regulation of rat interstitial collagenase mRNA expression and an inhibition of α1(I) collagen mRNA expression compared to the adhered gel.

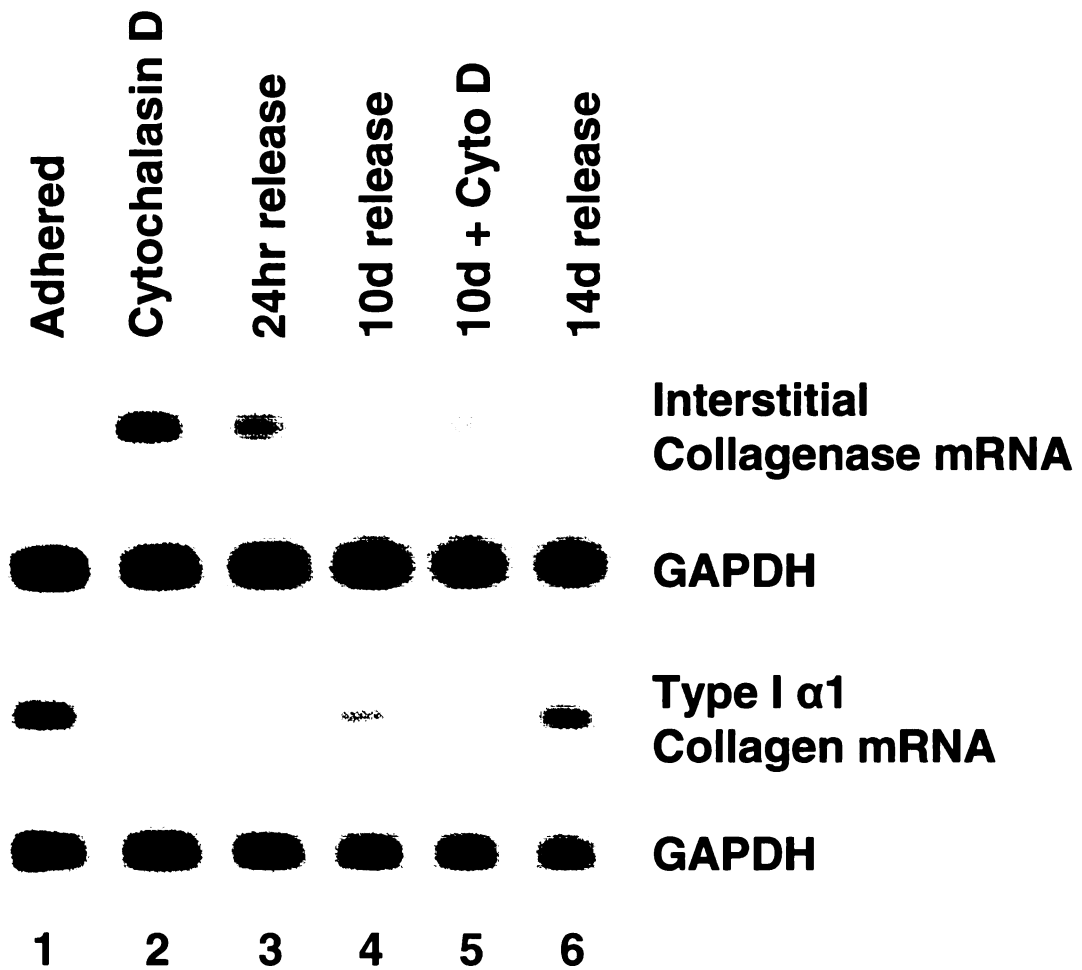

Figure 2.4 Representative Northern blot analysis of rat interstitial collagenase and α1(I) collagen with GAPDH as a control. Lanes represent 1) adhered to dish for 24 hours, 2) cytochalasin D for 24 hours, 3) 24 hours contraction, 4) 10 days contraction, 5) 10 days contraction plus cytochalasin D for additional 24 hours, 6) 14 days contraction.

Following release and 10 days of contraction (lane 4) the gels became more condensed and the cells demonstrated a down-regulation of interstitial collagenase mRNA expression and an up-regulation of α1(I) collagen mRNA expression (compared to actively contracting gels (lane 3)). The disruption of the cytoskeleton with cytochalasin D following 10 days of gel contraction (lane 5) produced the same mRNA expression pattern seen following cytoskeletal alteration in adhered gels (lane 2). This demonstrated that the cells still possessed the ability to respond to cytoskeletal alteration after 10 days. In gels that were released and allowed to contract for 14 days (lane 6), the gels reached an asymptotic contraction (based on previous studies (Arnoczky et al. 2004)) and the mRNA expression of rat interstitial collagenase and α1(I) collagen was similar to the adhered gel.

Contraction and Physical Alteration of the Cytoskeletal Tension

As noted previously, the tendon seeded collagen gels began to contract upon release from their individual culture dishes. However, instead of contracting into dense beads the gels contracted around the 3-point traction devices forming equilateral triangular structures comprised of bands of condensing collagen gel (Figure 2.5). Removal of the traction devices (and the opposing tractional resistance they provided) permitted further contraction of the gels (Figure 2.5).

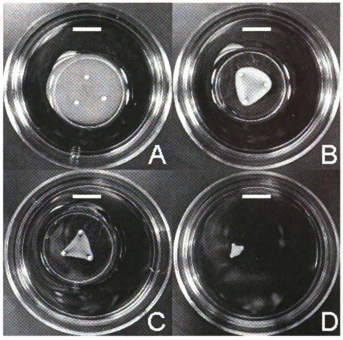

Figure 2.5 Photograph showing a representative gel with a 3-point traction device in place immediately after release **(A)** and after 24 hrs **(B)** and 10 days **(C)** of gel contraction around the three pins. Removal of the traction devices (and the opposing tractional resistance they provided) permitted further contraction of the gels **(D)**. (Scale bar = 10 mm).

In each of the groups examined, it was apparent that there was a reciprocal relationship between the rat interstitial collagenase mRNA and α1(I) collagen mRNA (Figure 2.6). As noted above, gels adhered to the culture dish for 24 hours (lane 1) produced no measurable expression of rat interstitial collagenase mRNA and a positive expression of α1(I) collagen mRNA. Releasing the gel and allowing it to contract around the 3-point pins for 24 hours (lane 2) caused an immediate up-regulation of rat interstitial collagenase and an inhibition of α1(I) collagen mRNA expression compared to the adhered gel. After releasing the gel and allowing a steady state of contraction around the 3-point pins after 10 days (lane 3) the cells regained similar mRNA expression to the adhered gel. The removal of the 3-point pins after 10 days of gel contraction (lane 4)

allowed additional contraction to occur (Figure 2.5). This apparent alteration in cell matrix interaction resulted in an up-regulation in the expression of interstitial collagenase and an inhibition of α1(I) collagen mRNA expression.

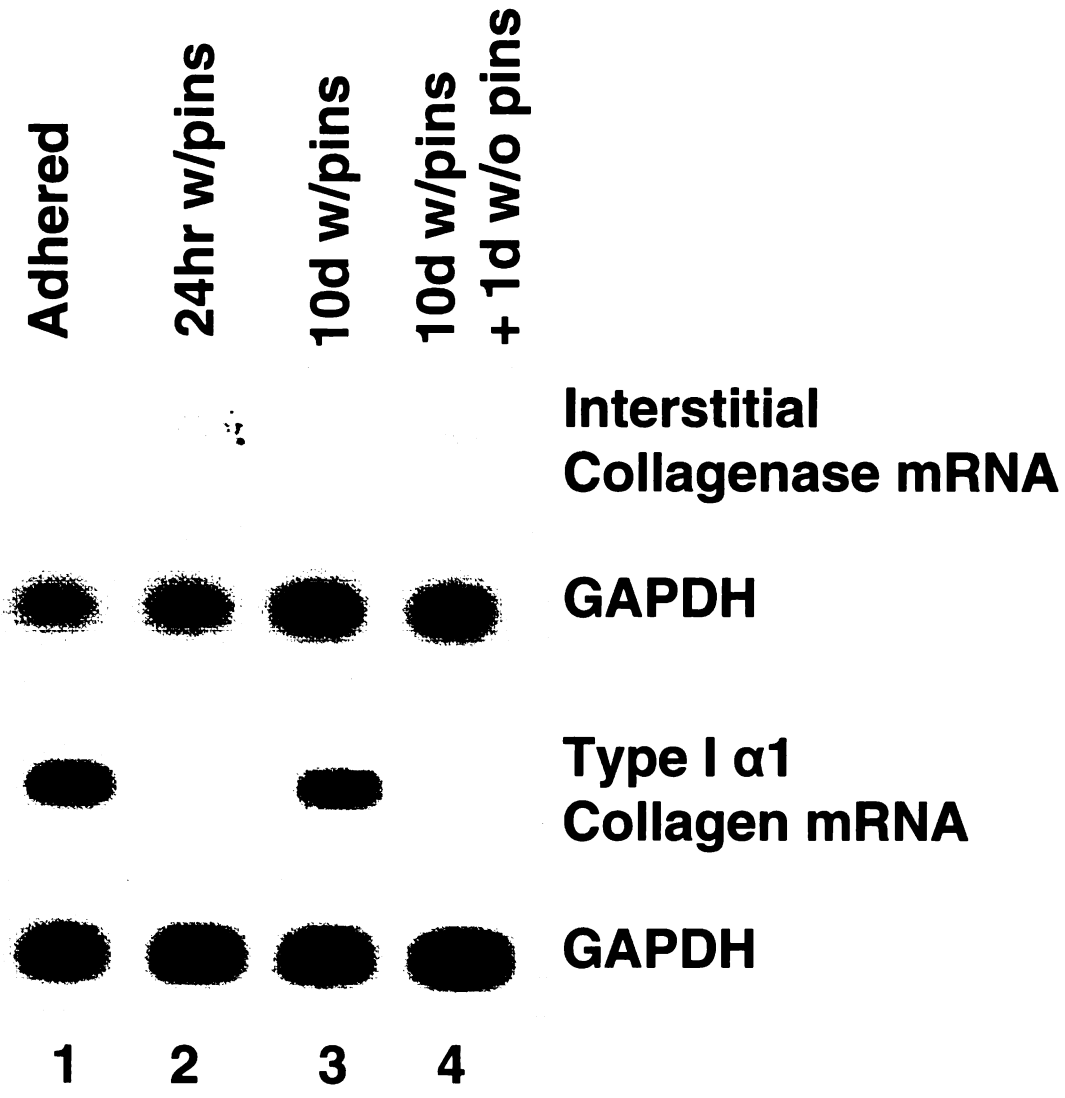

Figure 2.6 Representative Northern blot analysis of rat interstitial collagenase and α1(I) collagen with GAPDH as a control. Lanes represent 1) adhered to dish for 24 hours, 2) 24 hours contraction around 3pt traction pins, 3) 10 days contraction around pins, 4) 10 days contraction around pins plus free contraction for an additional 24 hours.

DISCUSSION

The results of the current study suggest that changes in cytoskeletal tension control a reciprocal expression of anabolic and catabolic genes by tendon cells. It has been suggested that the cellular regulation of biological function lies in the ability of cells to sense, generate, and balance mechanical forces (Chicurel et al. 1998). This mechanoresponsiveness has been shown to be mediated through a tensegrity apparatus comprised of the cell's cytoskeleton as well as its attachment(s) to the extracellular matrix (Chen and Ingber 1999; Ingber 1991; Ingber 1997; Kolodney and Wysolmerski 1992; Pourati et al. 1998; Ralphs et al. 2002; Roy et al. 1999; Tomasek et al. 1992). Previous studies have shown that cells can generate an internal tension within their cytoskeleton by way of an actomysin filament sliding mechanism (Dickinson et al. 1994; Skalak et al. 1994). This mechanism allows a cell to maintain a constant cytoskeletal tension in response to changes in external loading (Brown et al. 1998; Sims et al. 1992; Takakuda and Miyairi 1996). This has been termed cytoskeletal homeostasis and is thought to be the mechanism by which a cell maintains a pre-set level of sensitivity to external loading (Brown et al. 1998).

In the current study, a previously described collagen gel matrix model system was used to examine the effect of cytoskeletal tension on gene expression (Brown et al. 1998; Brown et al. 1996; Eastwood et al. 1994; Eastwood et al. 1998; Eastwood et al. 1996; Fringer and Grinnell 2001; Grinnell 1994; Grinnell 1999; Grinnell et al. 1999; He and Grinnell 1994; Lee et al. 1993; Lin and Grinnell 1993; Rosenfeldt et al. 1998). In this system, tendon cells seeded into the collagen gels were able to establish a cytoskeletal tensional homeostasis through an isometric contraction against collagen gel matrices left

attached to their culture dishes. This was characterized by the presence of organized stress fibers within the cytoskeleton and an up-regulation of the anabolic gene (α1(I) collagen). Loss of cytoskeletal organization through chemical disruption or a detachment of the gel resulted in an up-regulation in the expression of the catabolic gene (interstitial collagenase) and an inhibition in the expression of the anabolic gene (α1(I) collagen). Previous studies have shown that alterations in cell shape, secondary to cell detachment (Aggeler et al. 1984; Unemori and Werb 1986), or loss of extracellular matrix tension produce an increase in interstitial collagenase expression (Arnoczky et al. 2004; Goomer et al. 1999; Lambert et al. 1992; Langholz et al. 1995; Lavagnino et al. 2005; Lavagnino et al. 2003; Loitz et al. 1989; Mauch et al. 1989; Prajapati et al. 2000; Unemori and Werb 1986) and a decrease in collagen production (Lambert et al. 1992; Mauch et al. 1989; Mochitate et al. 1991; Prajapati et al. 2000; Unemori and Werb 1986).

Detachment of the tendon cell seeded collagen gels was followed by a contraction of the matrices by the cells, either on themselves or around the three point traction devices. The phenomenon of collagen gel matrix contraction by fibroblasts has been documented in numerous studies (Brown et al. 1998; Brown et al. 1996; Eastwood et al. 1994; Eastwood et al. 1998; Eastwood et al. 1996; Fringer and Grinnell 2001; Grinnell 1994; Grinnell 1999; Grinnell et al. 1999; He and Grinnell 1994). The ability of the cells to re-align their extracellular matrix by way of an internal contractile mechanism has been implicated in the process of wound contracture and connective tissue morphogenesis (Grinnell 1994; Grinnell 1999; Grinnell 2000; Harris et al. 1981; Stopak and Harris 1982). In both instances, interstitial collagenase is thought to play a critical role in the remodeling process of the extracellular matrix (Sternlicht and Werb 2001;

Werb and Chin 1998). During contraction of the gels the cells are thought to exert an isotonic force against the contracting extra-cellular matrix (Grinnell 2000). As contraction slows and the remodeling matrix is able to produce an opposing external force (either by contracting against fixed points or condensing upon itself) the forces exerted by the cells becomes isometric and a homeostatic internal tension develops (Brown et al. 1998; Grinnell 2000).

In the current study, interstitial collagenase expression was partially inhibited after 10 days in the freely contracting collagen matrices as the gels condensed and began to provide resistance to the cellular tension and completely inhibited after 14 days when the freely contracting gels reached an asymptotic level of contraction (Arnoczky et al. 2004). Contraction of the gels around the 3-point traction devices at 10 days resulted in a complete inhibition of interstitial collagenase. Removal of the traction devices permitted additional gel contraction and initiated an immediate up-regulation of interstitial collagenase expression. In all cases, a decrease in interstitial collagenase expression was accompanied by a reciprocal increase in α1(I) collagen expression. Thus, the cells were able to return to their baseline level of gene expression (seen in the attached collagen gel matrices) by altering their local extracellular environment through contraction of the gels.

The ability of cells to actively "tune" their microenvironment in order to manipulate the degree to which they sense strain is thought to be the mechanism by which tissues adapt to large variations in mechanical loading over time (Frost 1987; Rubin et al. 1999). A recent study in bone suggested that osteocytes can actively modulate their response to external loading by regulating their connections to the extracellular matrix in both a positive (collagen) and negative (collagenase) manner

(Rubin et al. 1999). It was theorized that through this "autoregulation" the osteocytes were able to adjust their exposure to strain stimulus through the regulation of the physical constraints of their own microenvironment (Rubin et al. 1999). This would permit a cell to maintain a "set-point" of activation with respect to the level of mechanical stress required to initiate a biologic response. This has been termed the "mechanostat set-point" in bone cells (Frost 1987) and a similar phenomenon may occur in tendon cells.

Of particular interest in this study was the apparent ability of the tendon cells to re-establish their base-line level of internal cytoskeletal tension (as evidenced by a return to baseline gene expressions) following the loss of opposing external forces offered by the collagen matrices following release. This suggests that tendon cells can play an active role in "recalibrating" their sensitivity to changes in external stresses. While this "recalibration" was accomplished by a gross reorganization and contraction of the pliable collagen gel matrices over a 10 day time period such alterations in cell-matrix interactions may be more localized (i.e. the pericellular matrix) and/or require more time (chronic exposure to altered matrix strains) in a mature connective tissue setting.

The current study only examined the response of tendon cells to alterations in cytoskeletal tensional homeostasis caused by a decrease in opposing external forces. The application of increased external forces (above the presumed set-point of the tendon cells) was not examined. A previous study has suggested that dermal fibroblasts can respond to increases in opposing external matrix strains by a reciprocal decrease in contractile force, which maintains a homeostatic tension (Brown et al. 1998). The same may hold true for tendon cells. However, the upper and lower limits of the external forces against which the cell can maintain tensional homeostasis is likely dependent on a myriad of factors

including cell type and local extracellular matrix composition as well as the frequency and rate of external stress application.

The relationship between internal cytoskeletal tension and gene expression would appear to be a key factor in understanding the ability (or inability) of cells to adapt their extracellular matrix to changing external stresses. The results of this study suggest that tendon cells can establish an internal cytoskeletal tension through interactions with their local extracellular environment. Alterations in this tension appear to control the expression of both catabolic and anabolic genes in a reciprocal manner. Although mechanotranduction signals are mediated through a variety of means (focal adhesions, integrins, cytoskeleton) (Banes et al. 1995), the current study focused on how cytoskeletal transformations lead to changes in gene expression. Additional studies are needed to examine the role of cell-matrix connections in establishing and maintaining tensional homeostasis in tendon cells and to determine the precise mechanical/physical threshold (set-point) that is required to activate various catabolic and anabolic genes.

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CHAPTER 3

Collagen Fibril Diameter Distribution Does Not Reflect Changes in the Mechanical Properties of *in vitro* Stress-Deprived Tendons

Michael Lavagnino
Steven P. Arnoczky
Katherine Frank[2]
Tao Tian

From the Laboratory for Comparative Orthopaedic Research
College of Veterinary Medicine, Michigan State University,
East Lansing, Michigan 48824, USA
[2]Kalamazoo College, Kalamazoo, Michigan 49006, USA

Lavagnino, M, Arnoczky, SP, Frank, K and Tian, T (2005) Collagen fibril diameter distribution does not reflect changes in the mechanical properties of in vitro stress-deprived tendons. J Biomech 38:69-75.

ABSTRACT

The purpose of this study was to determine if an association exists between the tensile properties and the collagen fibril diameter distribution in *in vitro* stress-deprived rat tail tendons. Rat tail tendons were paired into two groups of 21 day stress-deprived and 0 time controls and compared using transmission electron microscopy (n = 6) to measure collagen fibril diameter distribution and density, and mechanical testing (n =6) to determine ultimate stress and tensile modulus. There was a statistically significant decrease in both ultimate tensile strength (control: 17.95+/-3.99 MPa, stress-deprived: 6.79+/-3.91 MPa) and tensile modulus (control: 312.8+/-89.5 MPa, stress-deprived: 176.0+/-52.7 MPa) in the *in vitro* stress-deprived tendons compared to controls. However, there was no significant difference between control and stress-deprived tendons in the number of fibrils per tendon counted, mean fibril diameter, mean fibril density, or fibril size distribution. The results of this study demonstrate that the decrease in mechanical properties observed in *in vitro* stress-deprived rat tail tendons is not correlated with the collagen fibril diameter distribution and, therefore, the collagen fibril diameter distribution does not, by itself, dictate the decrease in mechanical properties observed in *in vitro* stress-deprived rat tail tendons.

INTRODUCTION

Tendons are composite aggregations of Type I collagen, elastin, proteoglycans, glycolipids, water, and cells. The collagen fibrils and their hierarchial arrangement are thought to play an important role in the tensile properties of the tendon (Kastelic et al. 1978; Viidik 1972). Previous research has documented changes in collagen fibril diameter distributions in tendons and ligaments as a result of aging (Derwin and Soslowsky 1999; Parry et al. 1978), stress (Cherdchutham et al. 2001; Zachos et al. 2002), stress deprivation (Binkley and Peat 1986; Nakagawa et al. 1989), genetically induced alterations in the extracellular matrix (Clark et al. 2001; Derwin and Soslowsky 1999), healing (Christel and Gibbons 1993; Frank et al. 1992), and anterior cruciate ligament graft remodeling (Frogameni et al. 1993; Oakes 1993).

Several studies have correlated a decrease in mean collagen fibril diameter with a loss of mechanical properties in healing and remodeling ligaments and tendons (Frogameni et al. 1993; LaPrade et al. 1997; Oakes 1993). Most authors attribute this to an increase in the production of small diameter collagen fibers associated with an increase in type III collagen synthesis (Shino et al. 1995). However, a recent *in vitro* study has suggested that the decrease in mean collagen fibril diameter seen in remodeling tendon grafts may, in part, be a result of enzymatic (collagenase) degradation of endogenous large collagen fibrils (Cunningham et al. 1999). In that study, rabbit medial collateral ligaments exposed to bacterial collagenase for 72 or 144 hours demonstrated a significant reduction in mean collagen fibril diameter.

Interstitial collagenase has been implicated in the loss of material properties associated with immobilization of ligament and tendons (Goomer et al. 1999; Loitz et al.

1989). Several experimental studies have documented an increase in interstitial collagenase mRNA expression following *in vivo* immobilization or *in vitro* stress-deprivation of ligaments and tendons (Arnoczky et al. 2004; Lavagnino et al. 2003; Majima et al. 2000). Thus, it is possible that interstitial collagenase may affect the mechanical properties of stress-deprived tendons and ligaments by enzymatically degrading endogenous collagen fibers.

The purpose of this study was to determine if an association exists between the tensile properties and the collagen fibril diameter distribution in a previously described *in vitro,* stress-deprived, rat tail tendon model (Arnoczky et al. 2004). In this model, *in vitro* stress deprivation has been shown to result in a significant increase in rat interstitial collagenase (MMP-13) mRNA and protein expression (Arnoczky et al. 2004). We hypothesized that *in vitro* stress deprivation for 21 days would result in a decrease in tensile properties (tensile modulus and failure stress) and that this change in material properties would reflect a decrease in mean collagen fibril diameter.

MATERIALS AND METHODS

Drugs and Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Ascorbate, gentamicin, and penicillin-streptomycin-fungizone solution were obtained from Gibco (Grand Island, New York, USA).

Rat Tail Tendons

Following institutional animal care and use approval, tendons were obtained for this investigation from the tails of two 13-month-old Sprague-Dawley rats, euthanized with sodium pentobarbital injection. The tendons were removed immediately after

euthanasia. Using a sterile scalpel blade, the tail was cut between coccygeal vertebrae at both the base and at the distal tip of the tail for a total length of approximately 120 mm. Tendons were gently teased from the distal portion of each tail with forceps and placed into a petri dish containing DMEM media supplemented with 10% FBS, antibiotic/antimycotic solution and Ascorbate. The rat tail tendons were then cut in half and paired into two groups: stress-deprived and control. The control group was immediately processed for either transmission electron microscopy (n = 6) or mechanical testing (n = 6). The stress-deprived tendon group were maintained in the above media incubated at 37°C and 10% CO_2 for 21 days, with media changed three times per week, before processing for either transmission electron microscopy (n = 6) or mechanical testing (n = 6). An additional 30 tendons were divided into control (0 time) and stress-deprived (21 days) groups for a Northern blot analysis to assay for MMP-13 mRNA expression.

Transmission Electron Microscopy

Tendons were placed in 4% paraformaldehyde for 24 hours, transferred to 4% formalin, and then sent to be cut and embedded for transmission electron microscopy (TEM). The tendons were cut in cross section three times, with the location of the cuts equally distributed along the length of the tendon. The cross sections (100 nm thick) of each fixed tendon were rinsed in 0.1M phosphate buffer then placed in 1% osmium tetroxide in 0.1M phosphate buffer for 3 hours. They were then dehydrated in graded ethanol solutions (30%, 50%, 65%, 75%, 95%, 100%), and transferred to propylene oxide. Tendons were then infiltrated with an Epon-type resin (Poly/Bed 812; Araldite, and dodenyl succinic anhydride [DDSA] in ratios of 5:4:12 [Polysciences, Inc,

Warrington, PA, USA]). The resin was infiltrated in three steps (50%, 75%, and 100% resin: propylene oxide). Each infiltration process was performed for 12 hours, and then the specimens were hardened at 60°C for 48 hours. Thin sections, three from the center of each rat tail tendon, were stained with aqueous uranyl acetate and lead citrate, and then scanned with a Philips 301 TEM (Philips Electronic Instrument Co. Roselle, IL, USA). Three random fields were photographed from each section at a magnification of x 19,000.

Image Analysis

All images were scanned using a Hewlett Packard ScanJet IIcx (Palo Alto, CA, USA), and then quantitatively analyzed using Scion Image Beta 4.0.2 software (Scion Corporation, Frederick, MD, USA). All fibrils within each scanned field were analyzed. To eliminate any potential error due to non-perpendicular cuts, the collagen fibril minor diameter was used to represent actual collagen fibril diameter. The area of each collagen fibril was measured and used to calculate the collagen fibril density, defined as the sum of the area of collagen fibrils in the image divided by the total image area.

Mechanical Testing

Tendons were frozen at -80°C in media until testing. At the time of testing, tendons were thawed to room temperature and a 5 mm portion of each tendon dissected and placed in a saline filled 12-well plate to measure initial tendon diameter with a calibrated microscope. The tendon diameter was determined by taking the mean of four measurements perpendicular to the long axis of the tendon and a circular cross section was assumed for the computation of initial area (Haut 1983; Haut 1985; Haut 1986; Rowe 1985). The remaining portion of the tendon was gripped at the ends with saw-tooth clamps for a 40 mm gage length. The portion of the tendon under each clamp was

first air-dried and placed between two pieces of emery board while the midsection (test area) was kept moistened with saline (Haut 1983; Haut 1985; Haut 1986). Since dehydration increases the strength of rat tail tendons by nearly 5 times (Betsch and Baer 1980), premature fracture at the grip was eliminated. The gripped tendon was then mounted onto a custom-made material testing system. The system was equipped with a 5 lb load cell (Sensotec, Columbus, OH, USA), a linear variable differential transformer (LVDT) (Lucas Schaevitz, Pennsauken, NJ, USA) to measure grip-to-grip tendon displacement, and a motion controller (Newport, Fountain Valley, CA, USA) to strain the tendons at a constant rate of 0.168 mm/s (~0.42% strain/s). Each tendon was preloaded to 10 g then loaded at the above rate to failure in a phosphate buffered saline (PBS) bath at room temperature. The load and displacement values were recorded using an analog-to-digital computer data acquisition system. Failure stress, tensile modulus, and failure strain were computed from the load-deformation data.

RNA Extraction and Northern Blot Analysis

A Northern blot analysis was performed to assay for MMP-13 mRNA expression after 21-days of stress deprivation in 30 tendons (15 control and 15 stress-deprived). Total cellular RNA was isolated from the rat tail tendons in each group (control and stress-deprived) by the acid guanidine thiocyanate-phenol-chloroform procedure (totally RNA kit, Ambion Inc., Austin, TX, USA) and pooled. The RNA samples were subjected to electrophoresis on 1.2% agarose gels containing 0.66M formaldehyde and MOPS, then transferred to a nylon membrane (Pierce Corp, Rockford, IL, USA) for 1 hour in TAE at 300 mA. Following transfer, the membrane was air-dried and UV cross-linked at 10 Joules/cm^2.

An MMP-13 probe (Lavagnino et al. 2003) and a human GAPDH cDNA control probe (Clontech Laboratories, Inc., Palo Alto, CA, USA) were labeled with biotin using the North2South Direct HRP Labeling and Detection Kit (Pierce Corp, Rockford, IL, USA). The RNA blots were hybridized with labeled probes (10ng/ml hybridization solution) at 55°C for 1 hour. The membrane was then washed with 40 mL (~ 0.5 mL per cm^2) 2x SSC/0.1% SDS at 55°C (3 x 5minutes) and washed with 40 mL (~ 0.5 mL per cm^2) of 2x SSC at room temperature. Following the wash procedure, the membrane was incubated with chemiluminescent working solution for 5 minutes and exposed to films for 5-10 minutes.

Statistical Analysis

Paired t-tests were used to determine any significant statistical difference between control and deprived tendons for the mean fibril diameter and density. A three factor ANOVA was used to determine if there were any differences in collagen diameter distribution between control and deprived tendons, with treatment and size as fixed factors and tendon as a random factor. Paired t-tests were also performed to obtain the statistical difference in both the tensile modulus and ultimate failure strength from the mechanical tests. Statistical significance was set at $p<0.05$ for all tests.

RESULTS

Image Analysis

Fibril distributions for normal rat-tail tendons exhibit a near equal distribution of both small (<100 nm 52.7%) and large (>100 nm 47.3%) fibrils. There was no significant difference between control and stress-deprived tendons in the number of

fibrils per tendon counted, mean fibril diameter, or mean fibril density (Figures 3.1-2, Table 3.1).

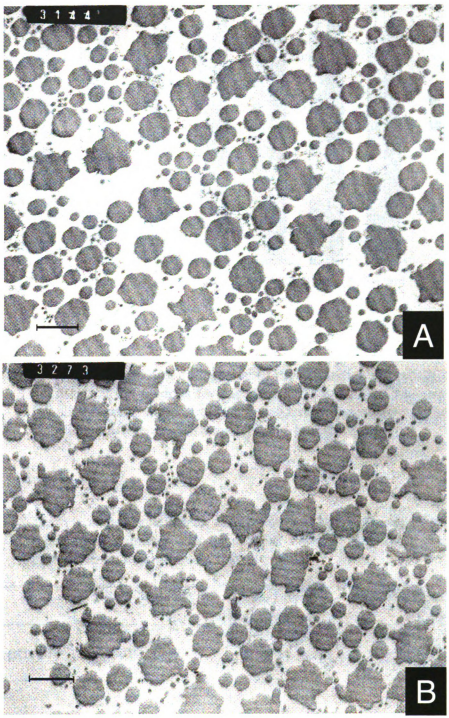

Figure 3.1 Representative transmission electron microscope image of **A**: control rat tail tendon cross-section and of **B**: a cross-section of a rat tail tendon stress-deprived for 21 days (x 19,000). Scale bar = 500 nm.

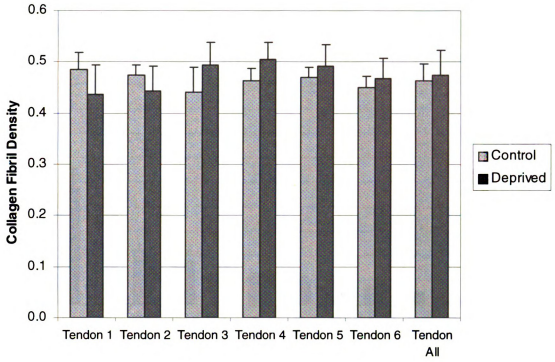

Figure 3.2 Histogram illustrating collagen fibril density in control and 21 day stress-deprived rat tail tendons for each of the six paired tendons measured and for all the control and stress-deprived tendons combined. There was no significant differences between tendons, $p>0.05$.

Table 3.1 Mean ± standard deviation for control and stress-deprived fibril number, mean fibril diameter and mean fibril density. Resulting p-value from paired t-test with significance set at $p<0.05$.

	Fibril Number	**Mean Fibril Diameter,** nm	**Mean Fibril Density,** nm^2/nm^2
Control	2951 ± 247	150.01 ± 10.9	0.464 ± 0.016
Stress-Deprived	2404 ± 224	153.65 ± 7.11	0.473 ± 0.029
p value	0.17	0.45	0.59

There was no significant difference in the fibril size distribution between control and deprived tendons within each fibril diameter bin (Figure 3.3).

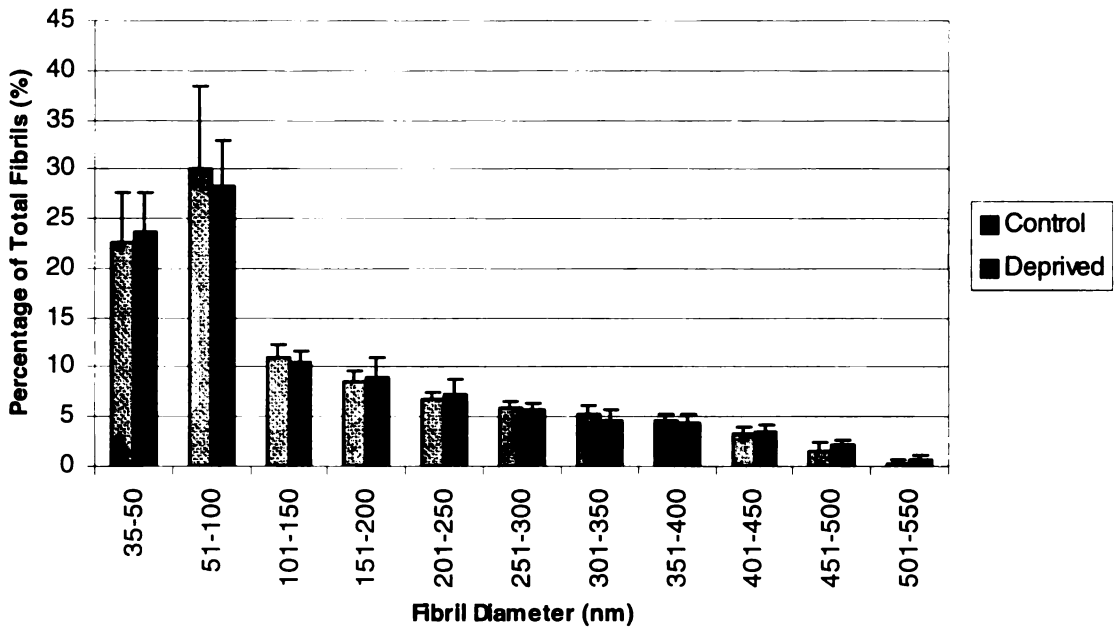

Figure 3.3 Histogram illustrating relative frequencies of collagen fibril diameters in control and 21 day stress-deprived rat tail tendons. There was no significant differences between tendons within each bin, $p>0.05$.

Mechanical Testing

There was a statistically significant decrease in both ultimate tensile strength (control: 17.95 ± 3.99 MPa, stress-deprived: 6.79 ± 3.91 MPa) and tensile modulus (control: 312.8 ± 89.5 MPa, stress-deprived: 176.0 ± 52.7 MPa) in the stress-deprived tendons compared to controls. A representative stress-strain curve from both groups is shown in Figure 3.4. A comparison of tensile modulus, failure stress, and failure strain for each pair of tendon samples is listed in Table 3.2.

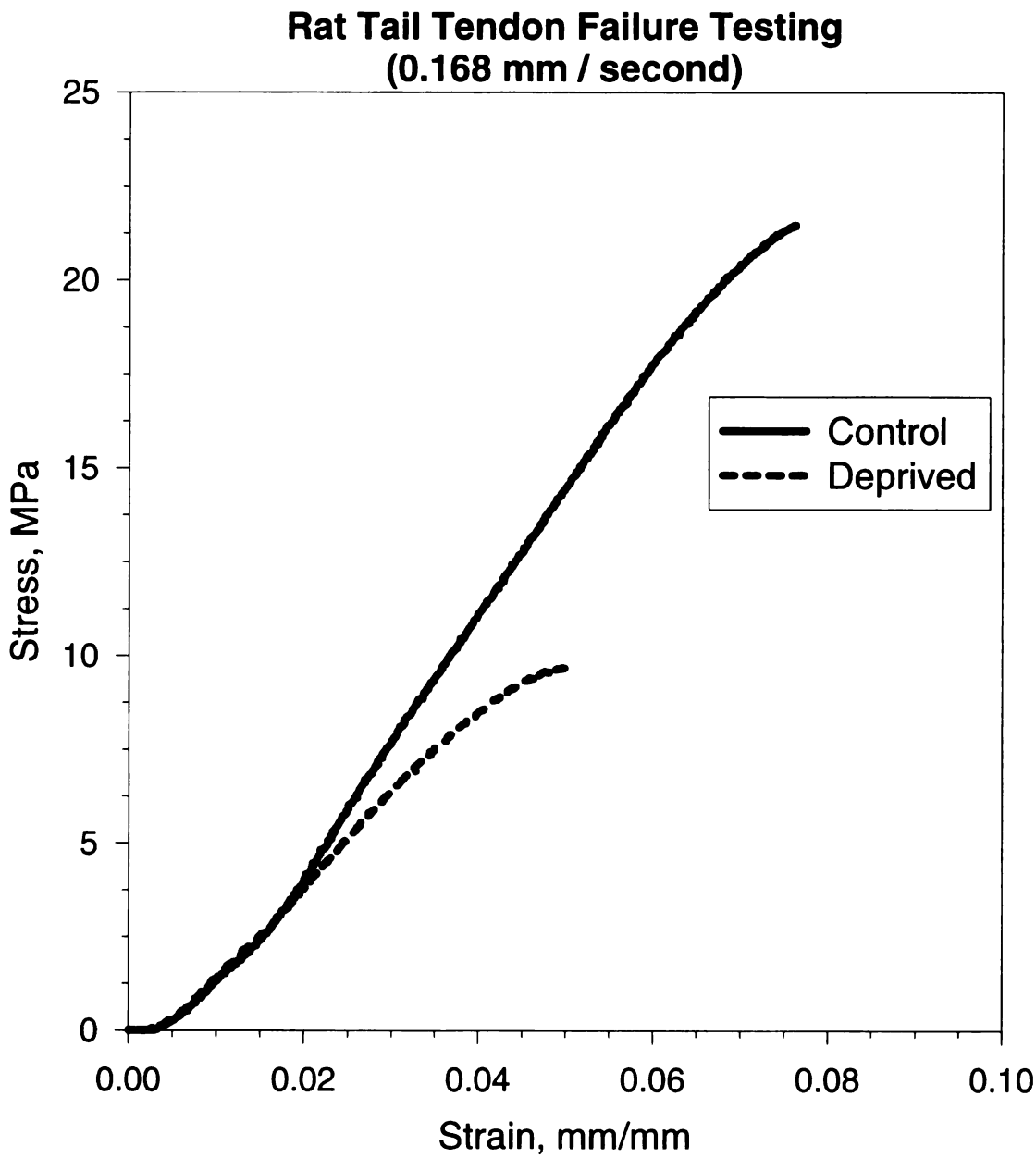

Figure 3.4 Representative stress versus strain curves from paired control and 21 day stress-deprived rat tail tendons of one fibril.

Table 3.2 Comparison of the cross-sectional area, tensile modulus, failure stress, and failure strain of control and stress-deprived rat tail tendons. †Cross-sectional area measurements were paired from the same tendon. The control tendon area was used for both groups to calculate failure stress. * significantly different than control specimens, $p<0.05$.

Tendon ID	Control				Stress-deprived			
	Cross Sectional Area (mm^2)	Tensile Modulus (MPa)	Failure Stress (MPa)	Failure Strain (%)	Cross Sectional Area (mm^2)	Tensile Modulus (MPa)	Failure Stress (MPa)	Failure Strain (%)
1	0.078	455	23.46	6.31	0.078	191	2.90	2.59
4	0.195	262	17.85	9.32	0.195	182	9.72	8.15
5	0.160	230	13.99	7.24	0.160	96	1.14	2.61
6	0.201	227	13.39	7.96	0.201	171	10.35	8.12
9	0.129	346	21.47	7.63	0.129	259	9.69	5.05
11	0.114	357	17.56	7.36	0.114	159	6.93	7.37
Avg.	**0.146**	**312.8**	**17.95**	**7.60**	**0.146**	**176.0***	**6.79***	**5.60***
SD	**0.048**	**89.5**	**3.99**	**0.99**	**0.048**	**52.7**	**3.91**	**2.57**

Northern Blot

The Northern blot gel demonstrated a complete absence of MMP-13 mRNA expression in the pooled control tendons. However, MMP-13 mRNA expression was clearly demonstrated in the pooled *in vitro* stress deprived tendons at 21 days (Figure 3.5).

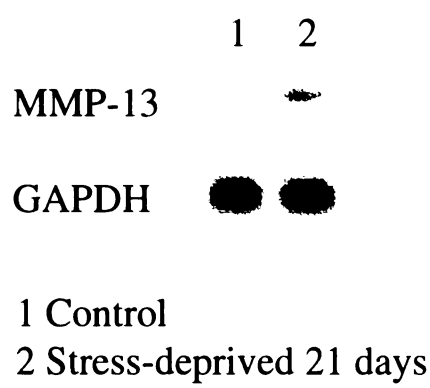

Figure 3.5 Northern blot gel illustrating the relative expression of MMP-13 mRNA expression in fresh control tendons (lane 1) and stress-deprived for 21 days (lane 2). GAPDH was used as an internal control.

DISCUSSION

Stress deprivation has been shown to have deleterious effects on the structural and functional properties of ligaments and tendons both *in vivo* and *in vitro* (Gamble et al. 1984; Hannafin et al. 1995; Majima et al. 1994; Noyes 1977). These cell mediated effects of stress deprivation are thought to produce multiple alterations in the biochemical and biomechanical character of these tissues including an increase in interstitial collagenase mRNA expression (Arnoczky et al. 2004; Goomer et al. 1999), an increase in collagen degradation and synthesis leading to an increase in collagen turnover and a decrease in collagen mass (Amiel et al. 1983), a decrease in proteoglycans and thus water content (Gamble et al. 1984; Woo et al. 1997), a decrease in ultimate failure stress and tensile modulus (Binkley and Peat 1986; Noyes 1977; Woo et al. 1982), and an increase in reducible collagen crosslinks (Akeson et al. 1987).

The results of this 21-day *in vitro* stress deprivation study demonstrate no significant difference in mean collagen diameter, mean collagen density, or collagen fibril diameter distribution between control and *in vitro* stress-deprived rat tail tendons. Previous 40-day *in vivo* stress deprivation studies have produced conflicting ultrastructural results. Binkley and Peat (Binkley and Peat 1986) found a significant increase in the proportion of larger fibrils and a significant decrease in the proportion of smaller fibrils in immobilized rat medial collateral ligaments compared with controls. Nakagawa et al. (Nakagawa et al. 1989) however, found significantly smaller mean collagen fibril diameter using a hindlimb disuse model of the rat Achilles tendon compared with control tendons. The disparity observed between the results of these studies as well as the results of the current study may be due to differences in

methodology, a variability in the degree of stress deprivation actually achieved, or differences in the tissues used. It is also possible that the increase in small diameter collagen fibrils observed in one *in vivo* study (Nakagawa et al. 1989) could actually be due to an anabolic production of new small diameter fibrils associated with tendon remodeling (Christel and Gibbons 1993; Frogameni et al. 1993; Oakes 1993) rather than a collagenase-induced catabolic deconstruction of large diameter fibrils as suggested by others (Cunningham et al. 1999). In remodeling tissues such as tendon grafts, small diameter fibrils are predominant and are thought to represent newly synthesized collagen (Shino et al. 1995).

The mechanical results of the current *in vitro* stress deprivation study agree with those from several *in vivo* immobilization studies (Binkley and Peat 1986; Noyes 1977; Woo et al. 1982), which demonstrated significant decreases in tensile modulus and ultimate tensile stress following immobilization. The absence of any significant difference in collagen fibril diameter distribution in the presence of significantly altered material properties seen in the present study suggests that collagen fibril diameter distribution does not solely determine the mechanical properties of rat tail tendons. The ultrastructural results of *in vivo* stress deprivation studies (Binkley and Peat 1986; Nakagawa et al. 1989) also lend support to this conclusion as they demonstrate that regardless of the collagen fibril diameter distribution (increase or decrease in small diameter collagen fibrils) the mechanical properties of these stress-deprived tissues diminished over time.

Although some studies have correlated changes in collagen fibril diameter with changes in mechanical properties of tendons (Parry et al. 1978) and ligaments (Binkley

and Peat 1986), other investigations have suggested that collagen fibril diameter distribution alone cannot predict the material and structural properties of tendons (Derwin and Soslowsky 1999; Derwin et al. 2001) and ligaments (Bay et al. 1993). Researchers have shown other factors beside collagen fibril diameter distribution are important for mechanical integrity and strength of the tendon (Clark et al. 2001; Derwin et al. 2001; Elliott et al. 2003; Haut 1985). Proteoglycans help to regulate the extracellular matrix assembly and structure and are thought to play a role in the structure function relation in tendons (Clark et al. 2001; Derwin et al. 2001; Elliott et al. 2003). An increased level of collagen crosslinking has been shown to inhibit the rate of collagenase activity (Woessner and Nagase 2002) and appears to increase the tensile modulus and reduce the strain to failure of collagen (Haut 1985; Thompson and Czernuszka 1995). Other factors that may play a role in determining the structure function relationship of tendons are the crimp pattern (Hansen et al. 2002), the length of the collagen fibril (Trotter and Wofsy 1989), the cell volume fraction or other sources of collagen irregularity (Derwin and Soslowsky 1999; Shrive et al. 1995) as well as the distribution and amount of proteoglycans and type I collagen (Kronick and Sacks 1994).

The results of the current study also demonstrate an increase in levels of rat interstitial collagenase mRNA expression following 21 days of *in vitro* stress deprivation. Other studies have shown increases in interstitial collagenase mRNA expression in tendons after 24 hours (Arnoczky et al. 2004) and 12 weeks (Goomer et al. 1999) of stress deprivation. A previous study from our lab using the same rat tail tendon model has demonstrated that MMP-13 mRNA expression correlated with MMP-13 protein synthesis (Arnoczky et al. 2004). Although MMP-13 expression in this study was clearly

present after 21 days of stress deprivation, there was no associated change in the collagen fibril diameter. Thus, the effect of cell-produced collagenase on collagen fibril diameter may not be as immediate or as profound as the previously published effects with bacterial collagenase (Cunningham et al. 1999). This is perhaps due to the impurities of other non-specific proteinases found within bacterial collagenase as well as its different mode of action in degrading type I collagen compared to interstitial collagenase (Woessner and Nagase 2002).

The failure strain of the normal control tendons in the current study was similar to that in other investigations (Haut 1985). The decrease in failure strain coupled with the decrease in tensile modulus observed following *in vitro* stress deprivation in the current study suggests a degradation in the properties of the collagen fibril itself rather than an increase in fibril sliding. However, the mechanism of stress deprivation and MMP-13 expression on the ultrastructural components of extracellular matrix and the subsequent correlation to the mechanical properties of stress-deprived rat tail tendons has yet to be determined.

Care must always be taken when comparing similarities and differences between *in vivo* and *in vitro* models at both the functional and molecular levels (Hart et al. 2002). It is likely that the level of stress deprivation achieved in the current *in vitro* system is more complete and more precisely duplicated when compared to the immobilization methods employed in other *in vivo* systems (Binkley and Peat 1986; Majima et al. 2000; Noyes 1977; Woo et al. 1982). While this may limit the ability to precisely compare the biochemical and physiological sequelae of altered stress conditions in the two systems (owing to the inability to precisely determine the levels of stress deprivation provided by

the various immobilization methods) we believe the *in vitro* stress deprivation model used in the current study is appropriate to test the hypothesis that changes in material properties are related to changes in collagen fibril diameter distribution.

In summary, the current study demonstrates that the changes in tensile properties observed in *in vitro* stress-deprived rat tail tendons are not related to changes in the collagen fibril diameter profile of the tissue. Furthermore, the results of the current study also suggest that, contrary to prior speculation (Cunningham et al. 1999), the presence of endogenous rat interstitial collagenase (MMP-13) does not produce an increase in small diameter collagen fibrils in these *in vitro* stress-deprived tissues. While *in vitro* stress deprivation in rat tail tendons has been shown to be associated with an increase in MMP-13 mRNA and protein expression and a decrease in tensile properties, the precise relationship between these sequela have yet to be determined.

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CHAPTER 4

Isolated Fibrillar Damage in Tendons Stimulates Local Collagenase mRNA Expression and Protein Synthesis

Michael Lavagnino
Steven P. Arnoczky
Monika Egerbacher
Keri L. Gardner
Meghan E. Burns

From the Laboratory for Comparative Orthopaedic Research
College of Veterinary Medicine, Michigan State University,
East Lansing, Michigan 48824, USA

Lavagnino, M, Arnoczky, SP, Egerbacher, M, Gardner, KL and Burns, ME (2006) Isolated fibrillar damage in tendons stimulates local collagenase mRNA expression and protein synthesis. J Biomech 39:2355-2362.

ABSTRACT

The etiology of repetitive stress injuries in tendons has not been clearly identified. While minor trauma has been implicated as an inciting factor, the precise magnitude and structural level of tissue injury that initiates this degenerative cascade has not been determined. The purpose of this study was to determine if isolated tendon fibril damage could initiate an upregulation of interstitial collagenase (MMP13) mRNA and protein in tendon cells associated with the injured fibril(s). Rat tail tendon fascicles were subjected to in vitro tensile loading until isolated fibrillar damage was documented. Once fibrillar damage occurred, the tendons were immediately unloaded to 100g and maintained at that displacement for 24h under tissue culture conditions. In addition, non-injured tendon fascicles were maintained under unloaded (stress-deprived) conditions in culture for 24h to act as positive controls. In situ hybridization or immunohistochemistry was then performed to localize collagenase mRNA expression or protein synthesis, respectively. Fibrillar damage occurred at a similar stress (41.13+/-5.94MPa) and strain (13.24+/-1.94%) in the experimental tendons. In situ hybridization and immunohistochemistry demonstrated an upregulation of interstitial collagenase mRNA and protein, respectively, in only those cells associated with the damaged fibril(s). In the control (stress-deprived) specimens, collagenase mRNA expression and protein synthesis were observed throughout the fascicle. The results suggest that isolated fibrillar damage and the resultant upregulation of collagenase mRNA and protein in this damaged area occurs through a mechanobiological understimulation of tendon cells. This collagenase production may weaken the tendon and put more of the extracellular matrix at risk for further damage during subsequent loading.

INTRODUCTION

It has been theorized that overuse injuries in tendons are caused by a failure of the tissue to adapt to repetitive microtrauma and a resultant deterioration of the extracellular matrix over time (Archambault et al. 1995). However, the precise magnitude and structural level of tissue injury that is required to initiate this degenerative cascade has not been determined. While some investigators have implicated overstimulation of tendon cells as a mechanobiological stimulus for inflammatory cytokine production and matrix degradation (Almekinders et al. 1993; Archambault et al. 2002; Tsuzaki et al. 2003)((Bhargava et al. 2004; Wang et al. 2003) these studies have been performed in monolayer culture and often include high strain magnitudes (Almekinders et al. 1993; Bhargava et al. 2004; Wang et al. 2003), long loading histories (Archambault et al. 2002; Bhargava et al. 2004; Wang et al. 2003), and the addition of biochemical factors (Archambault et al. 2002; Tsuzaki et al. 2003). Thus, the clinical relevance of such studies is unclear.

Recent investigations have demonstrated that fibroblasts are capable of establishing a cytoskeletal tensional homeostasis through interactions with their local extracellular environment (Brown et al. 1998; Lavagnino and Arnoczky 2005). This internal cellular tension has been shown to regulate gene expression in tendon cells and establish the cell's "calibration point" (Lavagnino and Arnoczky 2005). Mechanical forces which exert additional tension (above and beyond this homeostatic calibration point) to the cytoskeleton will elicit an anabolic response gene, while an absence of mechanical stimuli (or a decrease below the homeostatic level) will elicit a catabolic gene response (Arnoczky et al. 2004; Lavagnino and Arnoczky 2005; Lavagnino et al. 2003).

A decrease in extracellular strain in tendons (below homeostatic levels) has been associated with an increase in the upregulation of interstitial collagenase and a subsequent decrease in the tensile properties of these tissues (Arnoczky et al. 2004; Lavagnino et al. 2005). Therefore, it is possible that mechanobiological understimulation of tendon cells, due to an alteration in cell-matrix interactions, could also be an inciting factor in the etiology of overuse injuries.

Previous biomechanical studies have suggested that isolated collagen fibril damage occurs near the end of the linear portion of the load deformation curves of ligaments and tendons (Kastelic et al. 1980; Viidik 1972; Woo et al. 1982). While this damage may not affect the ultimate tensile strength of the tissues (Panjabi et al. 1996) it could alter the cell matrix interactions within the damaged portion of the tendon. The alteration of cell matrix interactions secondary to isolated fibrillar damage could cause a mechanobiological understimulation of tendon cells which has been shown to result in an up-regulation of collagenase mRNA expression and protein synthesis (Lavagnino and Arnoczky 2005). This, in turn, could weaken the tendon and put more of the extracellular matrix at risk for further damage with subsequent loading (Lavagnino et al. 2005). A recent study found that a general pattern of collagen deterioration and tissue degeneration was common to both ruptured and tendinopathic tendons suggesting a common, but as yet unidentified, cell mediated, pathological mechanism acting on both of these tendon populations (Tallon et al. 2001).

The purpose of the current study was to induce an isolated fibrillar failure in an *in vitro* rat tail tendon model system and determine if isolated fibrillar failure results in an up-regulation of collagenase mRNA expression and protein synthesis. The hypothesis

was that a mechanobiological understimulation of tendon cells secondary to isolated fibrillar damage in rat tail tendons would result in a local up-regulation in interstitial collagenase mRNA expression and protein synthesis in these cells.

MATERIALS AND METHODS

Rat Tail Tendon

Following institutional animal care and use approval, tendon fascicles were obtained from the tail tendons of adult Sprague-Dawley rats. The fascicles were removed immediately after euthanasia. Using a sterile scalpel blade, the tail was cut between coccygeal vertebrae at both the base and at the distal tip of the tail. Tendon fascicles were gently teased from the distal portion of each tail with forceps and then stored for less than four hours in a 100mm culture dish containing Dulbecco's Modified Eagle medium (DMEM) supplemented with an antibiotic/antimycotic solution until induction of fibrillar damage testing. During the fibrillar damage testing, the tendon fascicles were maintained in a complete media solution of DMEM supplemented with 10% fetal bovine serum, antibiotic/antimycotic solution, and Ascorbate at room temperature. Ten rat tail tendon fascicles from ten different rats were used to create the isolated fibrillar damage. To confirm that collagenase mRNA expression and protein synthesis was due to fibrillar damage and a resulting lack of mechanobiological stimulus, additional rat tail tendon fascicles were either processed immediately after harvest as negative controls (n=10) or maintained under non-loaded (stress-deprived) conditions in tissue culture for 24 hours as positive controls (n=10).

Induction of Fibrillar Damage

At the time of testing, a single rat tail tendon fascicle was mounted with a small amount of slack in a custom-designed, low-load, tensile testing apparatus (Arnoczky et al. 2002). The device was equipped with a 5 lb load cell (Sensotec, Columbus, OH, USA) and a linear variable differential transducer (LVDT) (Lucas Schaevitz, Pennsauken, NJ, USA) to measure grip-to-grip tendon displacement. Observations and fascicle diameter (268 ± 72 μm) measurements were made using a stereomicroscope (Olympus, Melville, NY, USA) with a 7x objective and recorded through the microscope using a CCD camera (Javelin Systems, Torrance, CA, USA). The initial fascicle length (40.4 ± 0.8 mm) was established by consecutively applying a known displacement (0.001 mm) through a computer controlled stepper motor until the slack was removed and the crimp pattern just began to diminish. The tendon fascicles were loaded starting from this crimped state (0% strain, 0 MPa) at a displacement rate of 20 μm/s until fibrillar damage occurred. Fibrillar damage in the fascicle was determined by visible fibril sliding and a change in the reflectivity of the damaged fibrils as a "crimp" pattern returned to these lax fibrils (Hansen et al. 2002; Kastelic et al. 1978; Viidik and Ekholm 1968). Previous studies have shown that reflective light can be used to delineate loaded (taut) from unloaded (lax) collagen fibers (Hansen et al. 2002; Kastelic et al. 1978; Viidik and Ekholm 1968). The alteration in reflectivity (and the associated reappearance of crimp) was used to delineate the location and extent of the damaged fibrils. Additionally, fibrillar damage was determined to occur when increases in strain produced a decrease in stress on the recorded stress-strain curve (Figure 4.1). Only those tendons in which isolated fibrillar damage could be documented on both imaging and the stress strain curve

were utilized. Immediately following visible fibrillar damage, the tendon fascicles were unloaded to 100 g (Figure 4.1) and maintained at that displacement while incubated at 37°C and 10% CO_2 for 24 hours. Following this 24 hour incubation period, the tendons were fixed in 4% paraformaldehyde at 4°C until processed for *in situ* hybridization or immunohistochemistry.

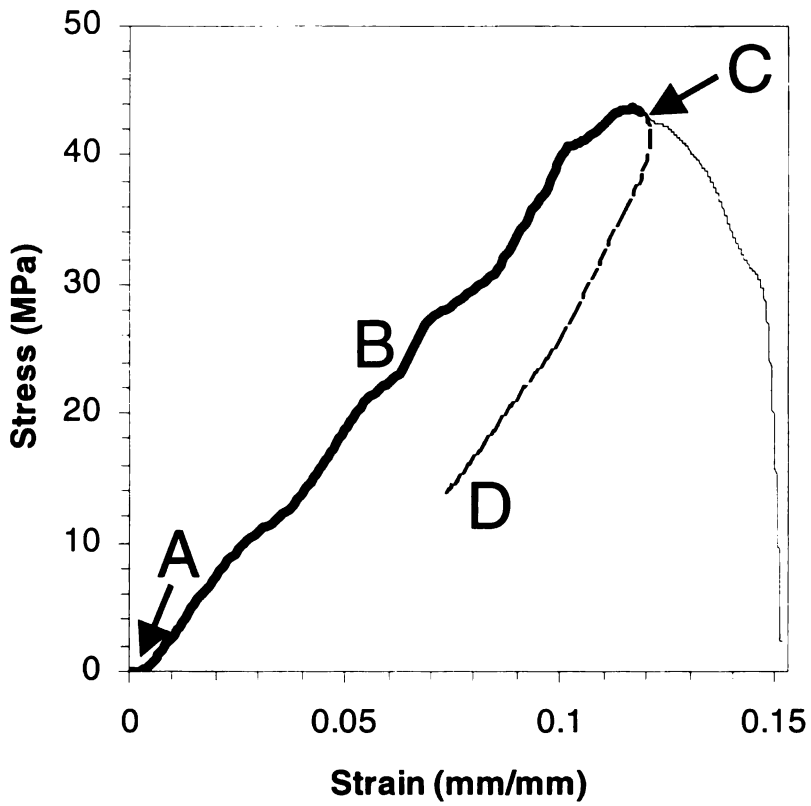

Figure 4.1 Representative stress-strain curve of a rat tail tendon fascicle (dark solid line) demonstrating the point at which fibrillar damage occurred (point C), and the unloading of the tendon to 100g (dashed line). A representative curve of the tendon loaded to failure displaying the negative slope in the stress strain curve that eventually ends in total tendon failure (Lavagnino et al. 2005) has also been included (light solid line). Points A-D on the stress-strain curve correspond to the images of the fascicle at those points in Figure 4.2A-D.

In situ hybridization

To determine the location of cells expressing rat interstitial collagenase (MMP-13), *in situ* hybridization was performed on the fixed tendon fascicles (5 injured, 5 fresh, 5 stress-deprived) to identify collagenase mRNA activity. The rat tail tendon fascicle was cut into 15 mm pieces to localize the injury site and reduce the tissue size to facilitate processing. The tendon fascicle segments were incubated in the following solutions: 0.2 N HCl (20 minutes), 3% hydrogen peroxide (20 minutes), RNase-free proteinase K (10 μL/mL for 10 minutes at 37°C) and 4% formalin in PBS. Subsequently, acetylation was performed with acetic anhydride in 0.1 M triethanolamine. The segments were rinsed in 2x SSC (1x SSC containing 150 mM NaCl and 15 mM sodium citrate) at 37°C. For hybridization, antisense and sense probes for rat interstitial collagenase were diluted in hybridization solution (50% deionized formamide, 2x SSC, 50μg/ml yeast RNA, 5mM EDTA, 0.2% Tween 20, 0.5% CHAPS and 100μg/ml Heparin) to a concentration of 2.5μg/mL, and incubated at 90°C for 3 minutes, then on ice for 5 minutes. The prehybridization was carried out at 60°C for 1 hour. The hybridization solution was then layered onto the fascicles and hybridized overnight at 37°C in a humid chamber. Posthybridization washes were performed at 60°C for 3x 15 minutes in 2x SSC with 50% formamide and 10% Chaps, then washed with 2x SSC for 2 minutes. The segments were incubated with block solution (Roche Diagnostics) for 1 hour at room temperature. The digoxigenin-labeled hybrids were detected by antibody incubation performed according to the manufacturer's instructions (Roche Diagnostics) with the following modifications. A 1:10 dilution of anti-digoxigenin (Fab) conjugated to Rhodamine was used for overnight incubation at room temperature. Then an extra washing step of 0.025% Tween

in Tris-buffered saline (pH 7.5) was performed. The fascicle segments were wet mounted on a glass slide for viewing with an Axiovert 200M fluorescent microscope (Carl Zeiss, Thornwood, NY, USA).

Preparation of RNA probe

A 1167-bp rat interstitial collagenase cDNA fragment was cloned into the pZErO^{TM}-2 vector (Invitrogen Life Technologies). The construct was then linearized by cutting it with EcoRI. *In vitro* transcription was performed using the appropriate RNA polymerases (T7 RNA polymerase for the anti-sense probe and Sp6 RNA polymerase for the sense probe) in the presence of digoxigenin (DIG)-linked UTP in the reaction mixture (DIG RNA Labeling Kit, Roche Diagnostics).

Immunohistochemistry

The rat tail tendon fascicles (5 injured, 5 fresh, 5 stress-deprived) were fixed in 4% paraformaldehyde for 24 hours, and rinsed in 70% ethanol and distilled water. The fixed tissues were permeabilized in 0.2% Triton X-100 for 1 hour and antigen retrieval was performed with an unmasking solution (H-3300, VECTOR Lab, Burlingame, CA, USA) for 1 hour at 65°C followed by cooling at room temperature for 20 minutes. Unspecific protein binding was blocked with a solution consisting of 2% horse serum, 1% BSA, 0.1% Triton X-100, and 0.05% Tween-20 in PBS for 1 hour. Anti- MMP-13 (Ab-5, NeoMarkers, Labvision Corp., Fremont, CA, USA) was applied to the fascicles overnight at 4°C at a dilution of 1:80. The primary antibody was detected with anti-rabbit Texas red (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:300. Tendon fascicles were mounted in Vectashield® mounting solution with DAPI (H-1200,

VECTOR Lab) and viewed under transmitted and fluorescent light on a Zeiss Axioplan microscope.

Statistical Analysis

The association between fresh control, injured, and stress-deprived rat tail tendon fascicles and interstitial collagenase gene expression and protein synthesis was evaluated for significance using a minimum chi-square test. Minimum chi-square values were calculated for both gene expression and protein synthesis among the three groups with a critical chi-square value of 5.99 ($p=0.05$) for significance.

RESULTS

The crimp pattern on the rat tail tendon fascicles, apparent at 0% strain (Figure 4.2A), disappeared with increasing tension (Figure 4.2B). Fibrillar damage was manifested as a visible sliding of fibrils and a resultant change in the reflectivity of the damaged fibrils (Figure 4.2C). This also coincided with a sudden decrease in stress on the stress-strain curve (Figure 4.1C). The precise location of the isolated fibrillar damage varied between samples. Fibrillar damage occurred at similar stress (41.13 ± 5.94 MPa) and strain (13.24 ± 1.94 %) values in all experimental tendons. This fibrillar damage occurred at approximately 79% of the failure strain of rat tail tendon fascicles loaded to failure in a previous study (Lavagnino et al. 2005). Upon unloading the tendon fascicles to 100 grams the crimp pattern reappeared within the area of fibrillar injury, but not in the remaining, uninjured, portion of the fascicles (Figure 4.2D). Isolated fibrillar damage did not appear to affect the ability of the remaining intact fascicle to support the 100g tensile loads. Thus, while fibrils within the tendon fascicle were damaged, the fascicle remained structurally intact.

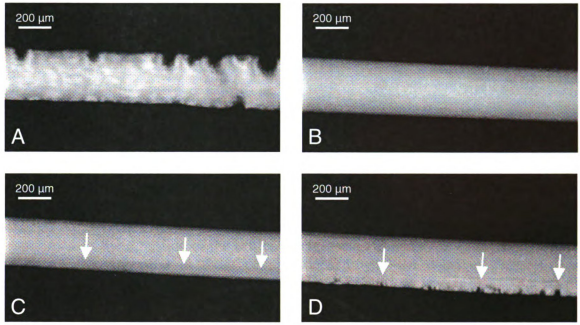

Figure 4.2 Images of a rat tail tendon fascicle at various points throughout the testing protocol: A) Prior to loading (the crimp pattern is clearly visible). B) During loading in the linear portion of the stress-strain curve demonstrating the elimination of the crimp pattern. C) Onset of fibrillar damage as manifested by a change in the reflectivity of the damaged fibrils (arrows). D) Unloading of the tendon to 100g and the reoccurrence of the crimp pattern within the damaged fibrils (arrows). (bar = 200 microns)

In situ hybridization of all the injured rat tail tendons indicated an up-regulation of MMP-13 mRNA in the cells within the damaged fibril(s) (Figure 4.3). Cells within the remaining, undamaged portion of the tendon fascicle showed no evidence of collagenase mRNA expression. Fresh control fascicles showed no evidence of collagenase mRNA expression while in the unloaded (stress-deprived) control samples, cells throughout the entire fascicle were positive for collagenase mRNA expression (Figure 4.4).

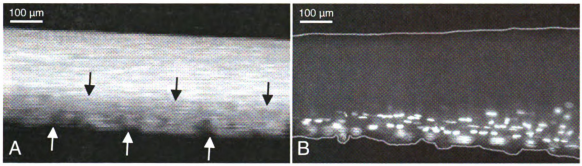

Figure 4.3 Representative images of a rat tail tendon fascicle following fibrillar damage. A) The presence of the crimp pattern on the bottom of the tendon fascicle (arrows) indicates the site of isolated fibrillar damage. B) *In situ* hybridization of the tendon fascicle reveals interstitial collagenase mRNA expression in those cells associated with the damaged fibril(s). The borders of the tendon fascicle are delineated by broken lines. (bar = 100 microns)

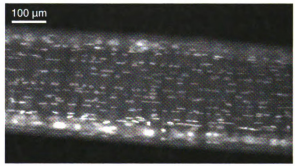

Figure 4.4 Representative image of a control (unloaded [stress deprived] for 24 hours) rat tail tendon fascicle demonstrating interstitial collagenase mRNA expression by cells throughout the entire fascicle. (bar = 100microns)

Immunohistochemical staining for interstitial collagenase protein produced results similar to that seen for collagenase mRNA. Cells within the damaged fibrils stained positive for collagenase protein while cells in the undamaged portion of the tendon fascicle were negative (Figure 4.5). Fresh control fascicles showed no evidence of collagenase protein synthesis while in the unloaded (stress-deprived) control samples, cells throughout the entire fascicle were positive for collagenase protein synthesis.

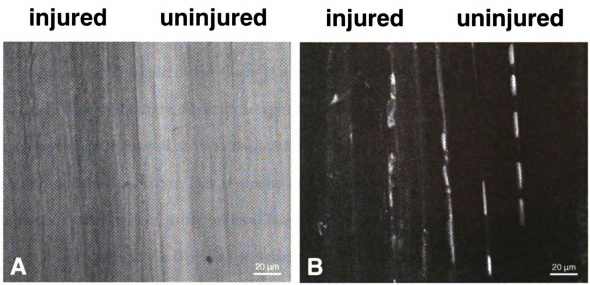

Figure 4.5 **A)** Representative photomicrograph of an injured rat tail tendon fascicle showing the damaged fibrils (denoted by the presence of crimp) immediately adjacent to uninjured fibrils. (bar = 20 microns)
B) Photomicrograph of the same field under fluorescent light demonstrating the positive (light gray) staining of MMP-13 protein in the cytoplasm of only those cells within the damaged fibrils. The nuclei have been counterstained with DAPI (white) to help identify the cells. (bar = 20 microns)

A significant association existed between control, injured, and stress deprived tendons and interstitial collagenase mRNA expression (Min. χ^2 = 14.14, p=0.001) and collagenase protein synthesis (Min. χ^2 = 14.14, p=0.001).

The absence of collagenase mRNA expression and protein synthesis in the fresh control fascicles suggests that the process of removing the rat tail tendon fascicles from the animal did not, in itself, contribute to the increase in collagenase gene expression and protein synthesis.

DISCUSSION

It is well known that increasing tensile loading alters the structure of tendons through the progressive loss of collagen crimp and the increase in fibril recruitment

(Diamant et al. 1972; Hansen et al. 2002; Kastelic et al. 1980; Viidik 1972). At the extremes of physiologic loading, fibril sliding and fibrillar damage occur prior to complete structural failure of the tissue (Kastelic et al. 1980; Viidik 1972; Woo et al. 1982). In the current study, fibrillar damage occurred at a similar strain (13.24 + 1.94%) in all experimental tendons and was documented by a sudden decrease in load as seen on the load deformation curve. This is similar to a recent study which documented fibril sliding in rat tail tendons using confocal laser microscopy (Screen et al. 2004). In that study, the initiation of fibril sliding and damage took place at a median value of 13% strain and was found to coincide with a decrease in tensile stress (Screen et al. 2004).

The strain at which fibrillar damage occurred in the current study was approximately 80% of failure strain previously reported for rat tail tendon fascicles (Haut 1985; Lavagnino et al. 2005). Studies in ligaments have shown that while loading the tissue to 80% of the failure deformation did cause isolated fibrillar damage, this isolated fibrillar damage had no effect on the mechanical properties above 80% deformation including failure load and failure deformation (Panjabi and Courtney 2001; Panjabi et al. 1999; Panjabi et al. 1996). The investigators suggested that the isolated injury could be sufficient to stimulate a biological response without sacrificing the gross mechanical behavior of the ligament (Panjabi and Courtney 2001).

The ability to produce isolated fibril failure within an otherwise intact tendon fascicle may be attributable to the multicomposite structure of the tissue (Kastelic et al. 1980; Viidik 1980). The sequential straightening and loading of crimped collagen fibrils, as well as interfibrillar sliding and shear between fibers and/or fibrils, produce a non-linear load-deformation behavior of tendons that may put certain fibrils "at risk" for

damage before others (Viidik 1980; Viidik 1990). Indeed, previous studies have demonstrated that individual tendon fiber and fibril failure begins to occur near the end of the linear range of the stress strain curve prior to complete failure (Viidik 1980; Viidik 1990). While the isolated fibril damage seen in the current study still permitted the remaining tendon to withstand loading (100g) within the linear range of the load deformation curve of the fascicles, the mode of fibril injury or the effect of this injury on the overall mechanical properties of the fascicles was not determined. Further studies are needed to elucidate these micro-injury mechanisms and their impact on tendon material and structural properties.

A previous study has shown that isolated fibrillar damage in tendons has been followed by a relaxation of the damaged fibrils (Knorzer et al. 1986). This relaxation (or laxity) of the damaged fibrils was identified by a change in the reflectivity of incident light by these lax collagen fibrils when compared to adjacent loaded fibrils (Hansen et al. 2002; Kastelic et al. 1978; Viidik and Ekholm 1968). The sudden change in light reflectivity (and the reappearance of crimp) in isolated collagen fibrils coincided with a decrease in strain on the stress strain curve confirming fibril damage.

In the current study, the reappearance of crimp within the damaged fibril(s) was observed immediately after injury suggesting that these fibrils were no longer transmitting load. The importance of mechanical stress on gene expression in tendon cells has been the subject of several recent investigations (Arnoczky et al. 2004; Lavagnino and Arnoczky 2005; Lavagnino et al. 2005; Lavagnino et al. 2003). These studies have shown that loss of a homeostatic tensile load on tendon cells results in an immediate upregulation of interstitial collagenase mRNA and protein synthesis

(Arnoczky et al. 2004; Lavagnino and Arnoczky 2005; Lavagnino et al. 2005; Lavagnino et al. 2003). In the current study, isolated fibrillar damage resulted in the immediate upregulation of collagenase mRNA expression in those tendon cells within the damaged fibril(s). This would suggest an altered cell-matrix interaction within the damaged portion of the fascicle. While this alteration (decrease) in mechanotransduction stimuli is most likely a result of damage to the extracellular matrix (e.g., collagen fibrils, collagen crosslinks, etc.) of the tendon fascicle and a subsequent loss of load-transmitting function, it could also be a result of damage to other components of the mechanotransduction pathway. Fibril sliding in loaded tendons produces strong local shear forces (Knorzer et al. 1986) which could damage cell-matrix adhesions (focal complexes, focal adhesions, or fibrillar adhesions) and/or alter the cytoskeleton. Indeed, a previous study has demonstrated that disruption of the cytoskeleton resulted in an immediate upregulation of collagenase mRNA expression in tendon cells within a loaded tendon (Arnoczky et al. 2004). Additional studies are needed to determine what, if any, portion(s) of the mechanotransduction pathway could be damaged by this fibrillar injury mechanism.

Collagenase expression, secondary to mechanobiological understimulation in tendons has been associated with a significant decrease in tensile properties (Lavagnino et al. 2005; Majima et al. 1994). Stress deprivation of rat tail tendons resulted in a significant increase in interstitial collagenase mRNA and protein expression as well as a decrease in both tensile modulus and tensile strength (Lavagnino et al. 2005). A clinical study examining matrix metalloproteinase activity in ruptured human tendons demonstrated a significant increase in interstitial collagenase activity when compared to

normal controls (Riley et al. 2002). This increase in collagenase activity was associated with a deterioration in the quality of the collagen network. Another study which examined the histopathology of ruptured and tendinopathic Achilles tendons suggested that while the ruptured tendons were significantly more degenerated than the tendinopathic tendons, the general pattern of tendon degeneration was common to both groups (Tallon et al. 2001). This implies a common pathological mechanism acting on both tendon populations (Tallon et al. 2001) and suggests that tendon degeneration is an active, cell mediated process that may result from a failure to regulate specific matrix-metalloproteinase activities in response to injury (Riley et al. 2002).

The results of the current study demonstrate that isolated fibrillar damage in tendon fascicles results in an upregulation of interstitial collagenase mRNA expression and protein synthesis in those tendon cells associated with the damaged fibril(s). This is thought to occur due to a mechanobiological *understimulation* of the tendon cells as a result of altered cell-matrix interactions. The upregulation of interstitial collagenase mRNA and protein by tendon cells throughout the unloaded (stress-deprived) control tendon fascicles further supports the role of mechanobiological understimulation of tendon cells in gene expression.

The resultant upregulation of interstitial collagenase mRNA expression and protein synthesis may weaken the tendon and put more of the extracellular matrix at risk for further damage with subsequent loading (Lavagnino et al. 2005). Therefore, it is possible that isolated collagen fibrillar damage may have more of an impact on long-term tendon health by altering homeostatic mechanotransduction interactions between the cell and the extracellular matrix rather than from any initial structural weakness of the tendon.

While any reparative response(s) secondary to the isolated fibrillar damage could not be examined in the *in vitro* system used in the current study, understanding the nature and extent of such responses would be critical in determining the ability of tendons to recover from this localized fibrillar damage.

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CHAPTER 5

A Finite Element Model Predicts the Mechanotransduction Response of Tendon Cells to Cyclic Tensile Loading

Michael Lavagnino[1]
Steven P. Arnoczky[1]
Eugene Kepich[2]
Oscar Caballero[1]
Roger C. Haut[2]

(1) Laboratory for Comparative Orthopaedic Research, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan 48824, USA
(2) Orthopaedic Biomechanics Laboratories, College of Osteopathic Medicine, Michigan State University, East Lansing, MI 48824, USA

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ABSTRACT

The importance of fluid-flow-induced shear stress and matrix-induced cell deformation in transmitting the global tendon load into a cellular mechanotransduction response has yet to be determined. A multiscale computational tendon model composed of both matrix and fluid phases was created to examine how global tendon loading may affect fluid-flow-induced shear stresses and membrane strains at the cellular level. The model was then used to develop a quantitative experiment to help better understand the roles of membrane strains and fluid-induced shear stresses on the biological response of individual cells. The model was able to predict the global response of tendon to applied strain (stress, fluid exudation), as well as the associated cellular response of increased fluid-flow-induced shear stress with strain rate and matrix-induced cell deformation with strain amplitude. The model analysis, combined with the experimental results, demonstrated that both strain rate and strain amplitude are able to independently alter rat interstitial collagenase gene expression through increases in fluid-flow-induced shear stress and matrix-induced cell deformation respectively.

1 INTRODUCTION

Cells are known to respond to physical signals by way of a mechanotransduction tensegrity system, which connects the cell nucleus to the extracellular matrix through a cytoskeleton (Banes et al. 1995; Brown et al. 1998; Ingber 1997). Deformation of the cytoskeleton, via membrane anchored attachment proteins (integrins), or stimulation of other transmembrane proteins (G-protein receptors, receptor kinases, mitogen-activated protein kinases) (Wang 2006) initiates a cascade of gene expressions activating catabolic and/or anabolic cell responses (Lambert et al. 1992; Lavagnino and Arnoczky 2005; Mochitate et al. 1991). For tendons, the importance of physical stimuli in maintaining homeostasis has been well documented (Hannafin et al. 1995; Lavagnino et al. 2005; Yasuda and Hayashi 1999). Recent *in vitro* studies have shown that stress deprivation of tendon cells *in situ* results in an immediate up-regulation of rat interstitial collagenase mRNA expression (Arnoczky et al. 2004; Lavagnino et al. 2003). Conversely, application of static stress (Arnoczky et al. 2004) or cyclic strain (Lavagnino et al. 2003) has been shown to inhibit interstitial collagenase mRNA expression in this *in vitro* tendon model through a cytoskeletally based mechanism. While this gene inhibition has been shown to occur in an amplitude and frequency, dose-dependent manner (Lavagnino et al. 2003), the effect of cyclic loading rate on gene expression has not been explored.

Previous studies in bone have demonstrated that both matrix deformation and fluid flow are important regulators of gene expression (Han et al. 2004; Mullender et al. 2004; You et al. 2000). Application of tensile strain to tendons leads to a progressive loss of collagen crimp, an increase in fiber recruitment, and a subsequent deformation of the extracellular matrix (Hansen et al. 2002; Kastelic et al. 1980). Tendon tensile strain has

also been shown to modulate tendon cell deformation in a dose-dependent manner (Arnoczky et al. 2002). Cellular deformation has also been implicated as a cell signaling mechanism in tendon cells through a calcium-based pathway (Arnoczky et al. 2007).

In addition to matrix deformation, experimental studies have demonstrated interstitial fluid flow in response to cyclic tensile loading of tendons (Hannafin and Arnoczky 1994; Helmer et al. 2006; Lanir et al. 1988). Fluid flow has been shown to control gene expression in a monolayer of tendon cells, presumably through a mechanotransduction mechanism, but without the significant calcium influx seen in deformed cells (Archambault et al. 2002). Therefore, it is possible that the dose-dependent inhibition of collagenase gene expression observed with increasing cyclic load amplitudes and/or frequencies may be related to concomitant increase in deformation and fluid flow induced shear stress on individual cells in the tendon (Lavagnino et al. 2003). However, the exact levels of fluid-induced shear stress on tendon cells *in vivo* have not been identified (Archambault et al. 2002).

While external loading is known to be an important regulator of bone metabolism, it has been suggested that fluid flow may actually play more of a role than matrix deformation in bone cell mechanotransduction (You et al. 2000). In tendons, a biphasic model has suggested the importance of distortional strain and hydrostatic stress as important mechanical stimuli regulating the composition of tendon and gene expression (Giori et al. 1993). This and other biphasic tendon models have successfully modeled global tendon properties in response to strain (Atkinson et al. 1997; Giori et al. 1993; Yin and Elliott 2004), but none have predicted local strains or fluid-induced shear stresses on the cell.

The mechanical response of tendon to load is determined by its solid (collagen matrix) and fluid components (Atkinson et al. 1997). Collagen fibers and their crimp formation are thought to play a significant role in determining the toe-in region of the nonlinear stress-strain response of tendon (Kastelic et al. 1980). Glycosaminoglycans have also been cited as strong determinants of mechanical properties due to their role in fibril-fibril binding (Redaelli et al. 2003) and their interaction with the fluid in the extracellular matrix (Robinson et al. 2004). The fluid content of a tendon, or its hydration, is known to alter the mechanical response of tendons to strain rate (Haut and Haut 1997). Although each of these components (extra-cellular matrix deformation and fluid flow) is known to play an important role in the mechanical response of tendon to load, their influence on cellular mechanotransduction is unknown.

Therefore, the purpose of this study was to create a multiscale computational tendon model composed of both matrix and fluid phases to examine how global tendon loading may affect stresses and strains at the cellular level. *We hypothesized that mechanotransduction signaling in tendon cells can result from either increases in fluid flow induced shear stresses or membrane strains on the tendon cells.* The model was developed to study the results of previous experiments (Lavagnino 2003) as well as to guide the development of new experiments to help better understand the roles of membrane strains and fluid-induced shear stresses on the biological response of individual cells.

2 MODEL DEVELOPMENT

A multi-scale modeling approach was utilized in this study to help predict the levels of fluid-flow-induced shear stress on cells and cell membrane strain generated under various amplitudes and rates of global tendon loading.

2.1 Global Model

The global model was based on the geometry and composition of a rat tail tendon (RTT). Tendons are made of three main components: type I collagen (70-80% of dry weight), an extrafibrillar matrix (proteoglycans, glycolipids, cells), and water (60-80% of wet weight) (Woo et al. 1997). Each of these components has mechanically significant functions in the response of tendon to load (Woo et al. 1997).

2.1.1 Collagen Fibers

Collagen fibers have been previously modeled as linear elastic (Diamant et al. 1972; Kastelic et al. 1980; Stouffer et al. 1985), bilinear elastic (Belkoff and Haut 1992; Hurschler et al. 1997; Kwan and Woo 1989), and linear viscoelastic (Johnson et al. 1996; Lanir 1980; Wang et al. 1997; Wilson et al. 2004). To represent the nonlinear effect of collagen recruitment in the current model, collagen fibers were simulated using axially oriented nonlinear spring elements attached at the nodes of the matrix elements, similar to the model of Wilson et al. (Wilson et al. 1997). The tissue strain at which tendon fibers became uncrimped and began load bearing was assumed to vary linearly in the radial direction, with the largest strain required at the RTT center and the smallest strain at the outer boundary of the RTT (Hansen et al. 2002). The collagen fiber distribution in tendon was assumed to be uniform with no variation in collagen fiber density. Crosslinks between collagen fibers are formed by secondary collagen fibers (types VI, XII, XIV)

and glycosaminoglycans. Although crosslinks are thought to play a role in the mechanical properties of tendon (Haut 1985; Puxkandl et al. 2002; Redaelli et al. 2003; Robinson et al. 2004), they are only indirectly (Poisson's ratio, low permeability) included in the current model.

2.1.2 Transversely Isotropic Matrix

Following the experimental demonstration of fluid exudation from tendon during cyclic tensile loading (Hannafin and Arnoczky 1994; Lanir et al. 1988), computational models have incorporated the effects of permeability and interstitial fluid flow (Adeeb et al. 2004; Atkinson et al. 1997; Butler et al. 1997; Chen et al. 1998; Yin and Elliott 2004). To adequately model fluid flow in the current model, the RTT matrix was assumed to be transversely isotropic in the fiber or 2-axis direction, with a zero pore pressure enforced on the outer boundary to allow free fluid flow out of the tendon. The transversely isotropic model, with Poisson's ratio greater than 0.5, has been measured in ligament and shown to be necessary to generate fluid exudation in response to tensile load (Adeeb et al. 2004; Hewitt et al. 2001; Yin and Elliott 2004). Orthotropic materials may have higher Poisson's ratios (>0.5) than isotropic materials, as long as thermodynamic constraints are met (Adeeb et al. 2004). A Poisson's ratio greater than 0.5 is required for volume loss with uniaxial tension, and this volume loss is presumably due to fluid exudation from tendon (Yin and Elliott 2004). The high Poisson's ratio in tendon may be due to the organization of fibers and their crosslinks and not because of high Poisson's ratios of the individual tendon components (Adeeb et al. 2004). The Poisson's ratio in this model was chosen to reflect the fluid exudation effect of the whole tendon.

2.1.3 Tendon Permeability

A major determinant of fluid flow is permeability, but the permeability of tendon is not known. However, it has been suggested that permeability is strain (Lai et al. 1981; Weiss and Maakestad 2006; Yin and Elliott 2004), porosity (Chen et al. 1998) and/or voids ratio (van der Voet 1997) dependent. Higher water content in the peripheral rim compared to the tendon core suggests that the porosity or voids ratio may also be depth dependent within a tendon (Wellen et al. 2005). The permeability in tendon is also believed to be anisotropic with a higher permeability along the fiber direction than across the fibers (Butler et al. 1997; Chen et al. 1998; Han et al. 2000). Although the directional dependence within tendon is known, the actual values are still in question. Therefore, for the current model, permeability and voids ratio were assumed to be uniform throughout the tendon.

2.2 Submodel

A submodeling function was utilized to provide the output from the global RTT model as boundary conditions in the cell model. The submodel consisted of an ovoid-shaped cell, a low-permeable cell membrane, pericellular and extracellular matrices, and nonlinear collagen fibers. Although tendon cells are often arranged in columns, the current model used a lone cell located on the outer boundary of the tendon. The outer boundary of the tendon was of interest, as cells in this area were assumed to be subject to the largest matrix deformations and fluid flows. In addition, a previous *in situ* study documented the cell nuclei deformation in this location following applied strain (Arnoczky et al. 2002). The membrane of the cell was assumed to have a low permeability, thus diverting fluid to flow around and not through the cell (Ateshian et al. 2007). Surrounding the cell and cell

membrane was the pericellular matrix. The tendon pericellular matrix appears similar in composition to that of cartilage pericellular matrix in that they both contain versican and type VI collagen (Ritty et al. 2003). In cartilage, the pericellular matrix significantly alters the mechanical environment of the cell and thus plays a role in mechanotransduction (Guilak and Mow 2000). In addition, the submodel includes the transversly isotropic extracellular matrix and nonlinear collagen fibers as described for the global model.

3 FINITE ELEMENT METHOD

3.1 Global Model

The RTT was assumed to be cylindrically shaped with symmetry along the radial and axial directions. Thus, a global, poroelastic model of the RTT was created 20mm long and 0.15mm in width (Figure 5.1). The RTT was divided into 300, 4-noded, axisymmetric elements (0.2mm x 0.05mm) using the commercial code ABAQUS 6.3 (ABAQUS, Inc. Providence, RI).

3.1.1 Collagen Fibers

To represent the nonlinear effect of collagen recruitment, collagen fibers were simulated using axially oriented spring elements attached at the nodes of the matrix elements with a bilinear collagen spring stiffness of 0N/mm during fiber straightening and 20N/mm after fiber straightening (Wilson et al. 1997).

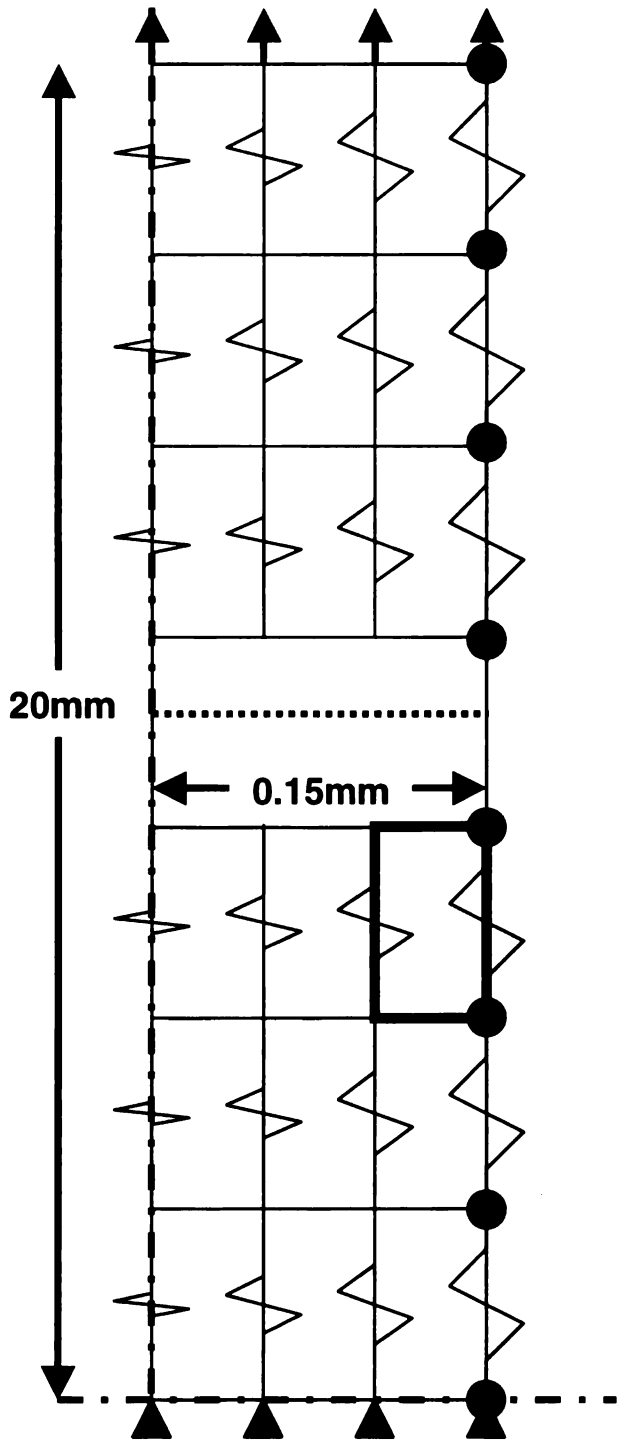

Figure 5.1 Axisymmetric global poroelastic model of the rat tail tendon (20mm x 0.15mm), divided into 300 4-noded axisymmetric elements (0.2mm x 0.05mm) with radially variant nonlinear spring elements attached at the nodes of the matrix elements (springs), zero pore pressure on the outer boundary (circles), constrained at the tendon center (triangles), and loaded at the tendon end as per previous experimental conditions (arrows). The darkened element boundary indicates the location of the submodel.

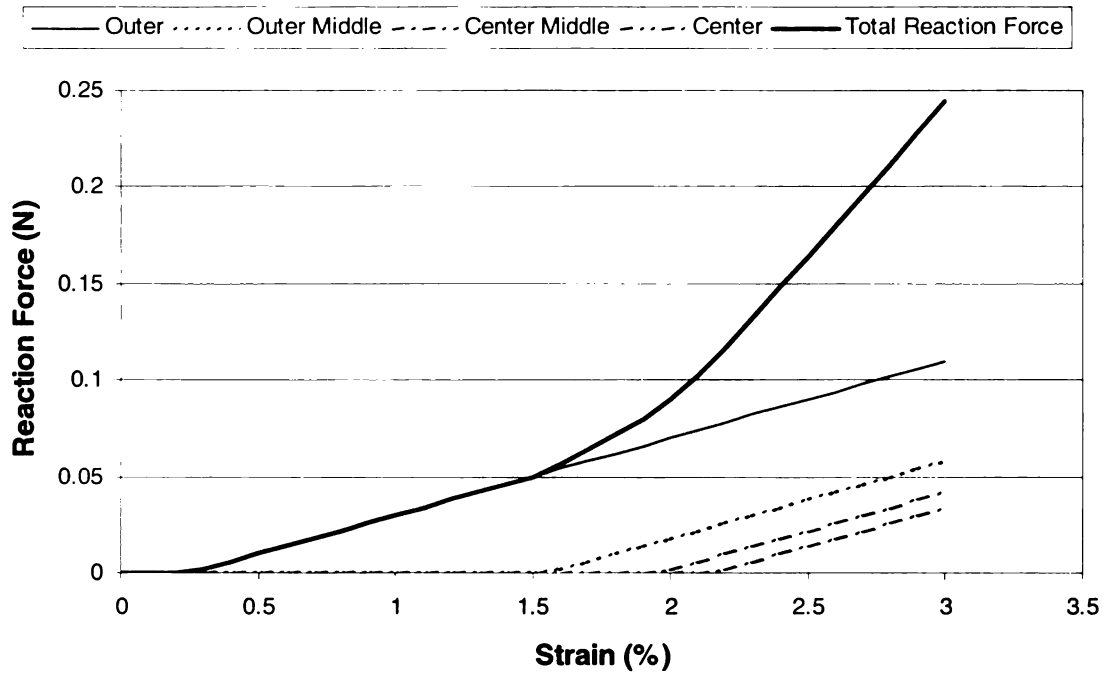

Figure 5.2 Reaction force (N) plotted against strain (%) to show the radial variation in fiber recruitment from the outer boundary to the inner or center that predicts the nonlinear response of the global tendon.

The tendon deformation at which the fiber became uncrimped and began load bearing varied in the radial direction, with the largest strain required at the RTT center and the smallest strain at the outer boundary of the RTT (Figure 5.2) (Hansen et al. 2002). A sensitivity analysis of the global tendon response to each parameter (data not shown) made it apparent that the collagen parameters (stiffness and uncrimping deformation) were dominant determinants of resultant force compared to matrix parameters. Therefore these collagen parameters were manually adjusted to fit the experimental stress-strain curve of a tendon loaded to 3% strain at 6% strain/minute. A constant fiber density of 4 columns of springs was used to mimic all collagen fibers.

3.1.2 Transversely Isotropic Matrix

To adequately model fluid flow in the current model, the RTT matrix was assumed to be transversely isotropic in the fiber or 2-axis direction. Material properties for the matrix were taken from the range of previous models and experiments on tendon and ligament (Table 5.1) (Adeeb et al. 2004; Atkinson et al. 1997; Gupta and Haut Donahue 2006; Haridas et al. 1999; Hewitt et al. 2001; Yin and Elliott 2004).

Table 5.1 Global matrix material properties.

Parameters	E_2 (MPa)	$E_1=E_3$ (MPa)	$\nu_{21}=\nu_{23}$	$\nu_{13}=\nu_{31}$	$G_{12}=G_{23}$ (MPa)	k (m^4/Ns)	Void Ratio
Matrix	1.0	0.0457	1.7	0.7	0.1	3.08e-14	2.0
Reference Range	0.0457-1.0	0.0457-1.0	0.7-2.73	0.3-0.9	0.157-5.0	5.5e-18-3.98e-14	1.0-2.33

(Adeeb et al. 2004; Atkinson et al. 1997; Gupta and Haut Donahue 2006; Haridas et al. 1999; Hewitt et al. 2001; Yin and Elliott 2004)

3.1.3 Boundary Conditions

Boundary conditions on the global model included zero pore pressures on the outer boundary to allow free fluid flow out of the tendon (Figure 5.1 circles) and constraint in the fiber direction at the RTT center to account for radial symmetry (Figure 5.1 triangles). The RTT was elongated with an applied displacement to a peak strain in the first cycle at the same applied strain rate as previous experiments: 1% strain at 2% strain per minute (0.017Hz); 1% strain at 20% strain per minute (0.17Hz); 3% strain at 6% strain per minute (0.017Hz) (Figure 5.1 arrows) (Lavagnino et al. 2003). Additionally the model was also run in the current study at 3% strain and 2% strain per minute (0.0056Hz) to help understand the individual roles of fluid-induced shear stress and membrane strain on interstitial collagenase mRNA expression of tendon cells.

3.2 Submodel

The submodel was not axisymmetric and was located in the midportion along the length of the global model on the outer boundary (Figure 5.1 darkened element) and composed of 4-node bilinear displacement and pore pressure (CPE4P) elements.

3.2.1 Submodel Components

A submodeling function was utilized to provide the output from the global RTT model (displacements, reaction forces, pore pressure) for boundary conditions in the cell model. The submodel was the size of the global element and it consisted of an ovoid-shaped cell, a low-permeable cell membrane, pericellular and extracellular matrices, and nonlinear collagen fibers (Figure 5.3).

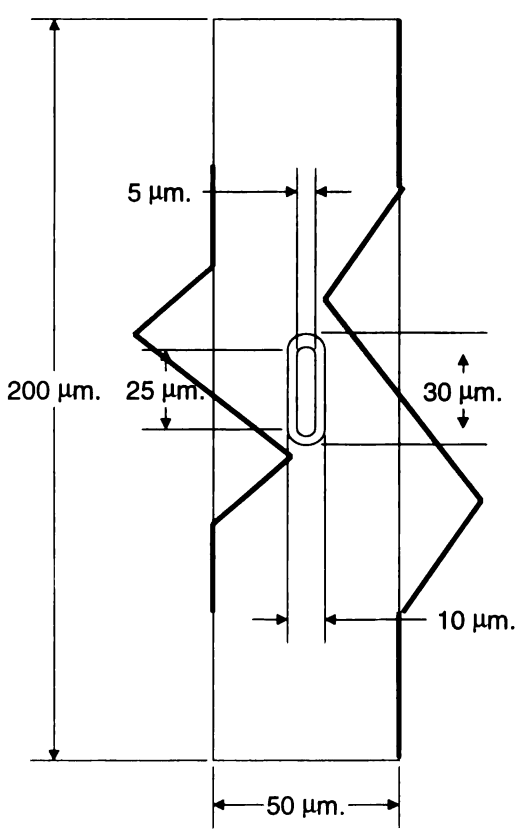

Figure 5.3 Submodel of the rat tail tendon, the size of a global element, composed of an ovoid-shaped cell, cell membrane, pericellular matrix (PCM), extracellular matrix (ECM), and collagen fibers.

The cell was chosen as an ovoid shape with a major axis length of 24μm and minor axis length of 4μm. The cell membrane in this model was used as a low-permeable barrier between the pericellular matrix and the cell to prevent fluid flow through the cell (Ateshian et al. 2007). The thickness of the membrane (500nm) was chosen in an attempt to maintain a similar element size as the rest of the submodel and thus maintain numerical stability. This same cell membrane thickness was also used in another submodeling study (Gupta and Haut Donahue, 2006). Two limitations associated with using a thicker membrane than normal (5-10nm) include altered permeability and stiffness values. Having a thicker cell membrane may make the membrane stiffer and thus less deformable. The permeability defined for the cell membrane in this study is higher by a factor of 20 than that previously prescribed to represent the permeability for a 500nm cell membrane (Ateshian et al. 2006). In taking the thickness of the cell membrane into effect we have potentially increased the cell stiffness and permeability and therefore predicted lower cell membrane deformation and fluid flow induced shear stress than if using a normal membrane size (10nm). Thus, by using a larger membrane thickness to help maintain element stability in the submodel and altering its theoretical permeability, the cell membrane deformations and fluid induced shear stresses were likely altered in an the same direction to preserve the relationship and help validate the overall conclusions of this first study. Some of these issues would necessarily have to be further investigated in future studies to develop this mechanotransduction computational effort. Surrounding the cell and cell membrane is the pericellular matrix with a 2.5μm thickness (Gupta and Haut Donahue 2006; Ritty et al. 2003). Linear poroelastic continuum elements were used to create the cell model with material properties taken

from the range of previous models (Table 5.2) (Guilak and Mow 2000; Gupta and Haut Donahue 2006). The transversely isotropic extracellular matrix, as described for the global model, surrounds the pericellular matrix. Nonlinear springs, as collagen fibers, connect only the central and outside edges of the cell model (Figure 5.3).

Table 5.2 Submodel material properties

Parameter	**E (MPa)**	**v**	**k (m^4/Ns)**	**Void Ratio**
Pericellular Matrix	1.0	0.490	3.924e-14	2.0
Cell Membrane	1.0	0.490	4.905e-19	2.0
Cell	0.5	0.069	4.415e-14	2.0
(Guilak and Mow 2000; Gupta and Haut Donahue 2006)				

3.2.2 Submodel Analysis

Coupled pore fluid diffusion and stress analysis were used to analyze the fluid flow velocities, stresses and strains within the model. Membrane strain was calculated by the change in the perimeter length divided by the original perimeter length as follows: $\varepsilon = \frac{\Delta \ell}{\ell}$. Fluid flow induced shear stress was calculated by the following equation $\tau = \mu \frac{du}{dv}$, where μ was the viscosity of the fluid (0.001Pa·s) and $\frac{du}{dv}$ was the change in the fluid velocity perpendicular to the surface of the cell (Gupta and Haut Donahue 2006).

4 EXPERIMENTAL METHODS

To compare the predicted cellular stresses and strains to the interstitial collagenase mRNA inhibition of tendon cells *in situ*, the following *in vitro* experiment was performed.

4.1 Cyclic Tensile Loading

After Institutional Animal Care and Use Committee approval was granted, rat tail tendons were harvested from adult Sprague-Dawley rats immediately after euthanasia. Using a sterile scalpel blade, the tail was cut between coccygeal vertebrae at both the base and at the distal tip of the tail for a total length of approximately 120 mm. Tendons were gently teased from the distal portion of each tail with forceps and maintained in DMEM media supplemented with 10% FBS, antibiotic/antimycotic solution and ascorbate incubated at 37°C and 10% CO_2. The rat tail tendons were divided into 6 groups as follows: *Group A*: 1% cyclic strain at 2% strain per minute (0.017Hz) for 24 hours; *Group B*: 1% cyclic strain at 20% strain per minute (0.17Hz) for 24 hours; *Group C*: 3% cyclic strain at 6% strain per minute (0.017Hz) for 24 hours; *Group D*: 3% cyclic strain at 2% strain per minute (0.0056Hz) for 24 hours; *Group E*: stress-deprived for 24 hours; *Group F*: fresh (0 hour) control. All experimental input parameters, except *Group D*, were the same as in previous studies (Lavagnino et al. 2003). Stress-deprived tendons were maintained in a culture dish in complete media under tissue culture conditions. Cyclic strain was applied to tendons in complete media under tissue culture conditions using a custom made, computer controlled, motor driven device (Lavagnino et al. 2003). While in the previous study Northern blots were used to show alterations in collagenase expression with various global loading parameters, in the current study matrix metalloproteinase (MMP)-13 (rat interstitial collagenase) mRNA expression was quantified using quantitative real-time polymerase chain reaction (Q-PCR).

4.2 Quantitative Polymerase Chain Reaction

At the end of each experimental period, ten tendons from each group were placed in 1.0mL of RNAlater™ (Qiagen, Valencia, CA) for a period of at least twenty-four hours at 4°C before processing. Total RNA was then extracted using the Qiagen RNEasy Kit (Valencia, CA) with the protocol provided for fibrous tissues. RNA (200-400ng), once concentrations were normalized, was converted into cDNA using the Invitrogen SuperScript III Reverse Transcription system (Carlsbad, CA). Real Time Quantitative PCR was performed using the TaqMan Gene Expression Assay from Applied Biosystems (ABI, Foster City, CA). Samples were run in a 96-well plate (20μl final volume per reaction) on an ABI 7500-Fast Q-PCR apparatus. The endogenous control used for all Q-PCR experiments was 18s rRNA. Results were analyzed using the Sequence Detection System software available from ABI. TaqMan probe and primer sets were obtained for MMP-13 (Rn01448197_m1) and 18s rRNA (Hs99999901_s1) from ABI's Gene Expression Assay database (http://allgenes.com).

5 RESULTS

5.1 Finite Element Results

The global model analysis closely predicted the nonlinear stress-strain response of the tendon to 3% strain at 6% strain/minute (Figure 5.4). In addition, the global model also indicated fluid flow in a radial direction leaving the tendon during tensile stretch (Figure 5.5). On the submodel level, this fluid was predicted to flow around the cell with the highest velocity occurring at the cell poles (Figure 5.6).

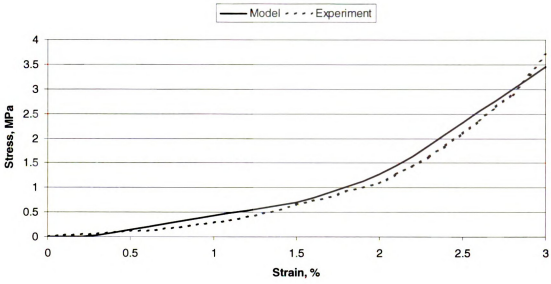

Figure 5.4 Comparison of tendon model to actual tendon stress-strain response (3% strain at 6% strain/minute).

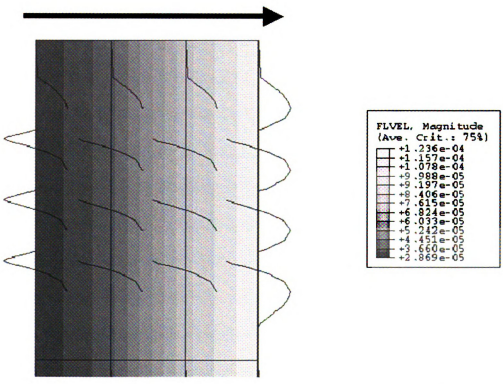

Figure 5.5 Plot of the fluid velocity magnitude (mm/s) showing fluid flow in the positive direction (arrow) out of the tendon (3% strain at 6% strain/min). The curved lines (springs) represent the collagen fibers.

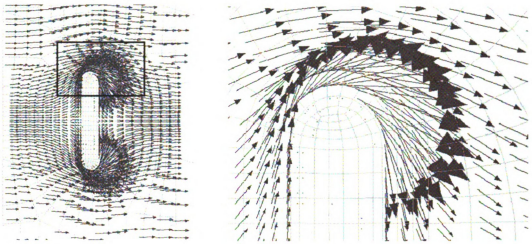

Figure 5.6 Plots of the fluid velocity resultant around the cell and out of the tendon (3% strain at 6% strain/min).

The tendon model predicted that increasing both the global strain rate (2% to 6% strain per minute) and strain amplitude (1% to 3%) (group A versus C) would result in an increase in both the maximum shear stress (142%) and cell membrane strain (191%) (Figure 5.7, Table 5.3). With an increase in the applied strain rate from 2% to 20% strain per minute at 1% global model strain (group A versus B), the model predicted a corresponding 124% increase in the maximum localized shear stress at the cell poles, but only a modest (5%) increase in cell membrane strain (Figure 5.7, Table 5.3). In contrast, the theoretical responses of the tendon model for inputs of 1% strain at 20% strain per minute (group B) versus 3% strain at 6% strain per minute (group C) generated similar maximum shear stress values (8% difference). However, the cell membrane strain levels were increased by 175%. The model was then exercised in an attempt to define an experiment to further explore the effects of membrane strain and shear stress. For a 3% strain at 2% strain per minute (group D), the model predicted a cell membrane strain within 8% of group C, with a maximum shear stress that was 145% less than group C (Figure 5.7, Table 5.3).

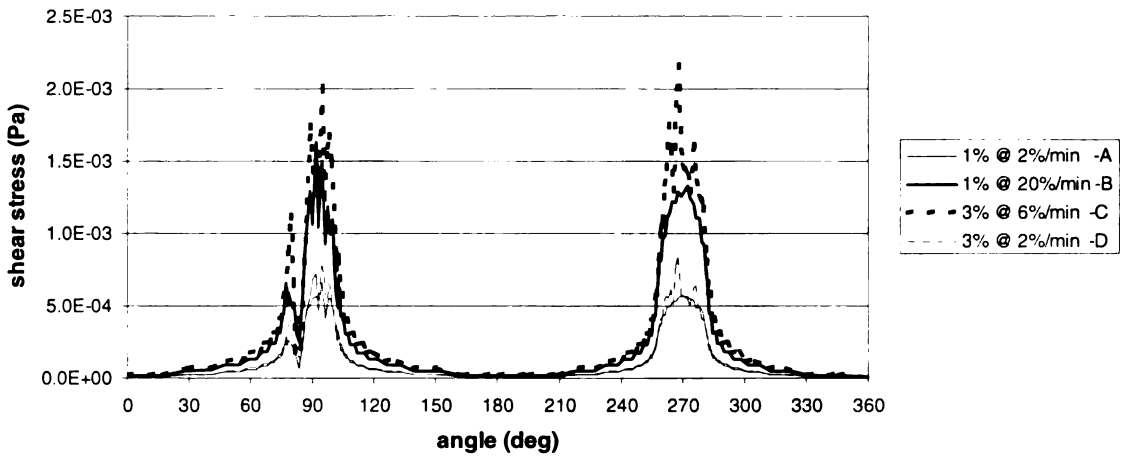

Figure 5.7 Graph of shear-stress induced by fluid flow. Note the marked increase in at the polar ends of the cell.

Table 5.3 Global model and submodel strain and shear stress values.

	Group A	Group B	Group C	Group D
Global Model Strain, %	1.00	1.00	3.00	3.00
Global Model Strain Rate, % strain/minute	2.00	20.0	6.00	2.00
Cell Membrane Strain, %	1.26	1.33	3.67	3.97
Maximum Shear Stress at 270°, mPa	0.414	0.927	1.004	0.409
Average Shear Stress, mPa	0.157	0.346	0.459	0.179
Collagenase Expression (Lavagnino et al. 2003)	Inhibited	Eliminated	Eliminated	NA

5.2 Q-PCR Results

The Q-PCR results from each group of tendons suggested direct correlations could be established between the levels of membrane strain and shear stress predicted in the model and the inhibition of collagenase mRNA. These data were calculated relative to the fresh control (0 hours) sample (Figure 5.8). Tendon cells exposed to a higher global strain rate and strain amplitude (group A versus C), expressed a significantly reduced amount of rat interstitial collagenase mRNA (Figure 5.8, Table 5.3). Increasing the global strain rate from 2% to 20% strain per minute at 1% global model strain (group A versus B) also significantly reduced this expression. On the other hand, increasing the amplitude of

global strain (1% to 3%), with a reduction of global strain rate from 20% to 6% per minute (group B versus C), had little effect on the expression of rat interstitial collagenase mRNA expression (Figure 5.8, Table 5.3). These data were thus suggestive that enhanced shear stress, rather than increased cell membrane strain was primarily responsible for inhibition of collagenase expression. Furthermore, it was seen that in comparing the results of group C to D, where the fluid induced shear stress on the cell was significantly reduced at 3% strain, the expression of rat interstitial collagenase mRNA was increased. Alternatively, if the Q-PCR results of group A versus D were compared, the collagenase expression was significantly reduced (Figure 5.8). This biological response of the tendon was associated with a theoretical increase in cell membrane strain and little change in the fluid-induced shear stress on the cell (Table 5.3).

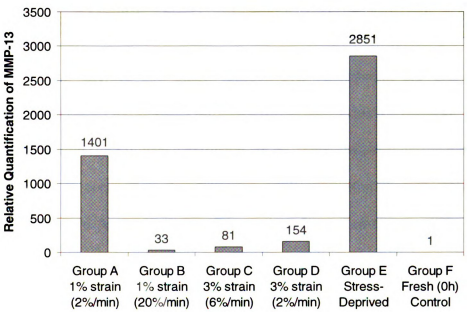

Figure 5.8 Gene expression levels of rat interstitial collagenase (MMP-13) as determined by Real-Time Quantitative PCR. All experimental samples were quantified relative to the fresh (0 hour) control.

6 DISCUSSION

The results of the initial study showed a strong association between the amount of fluid-flow-induced shear stress on the cell and the inhibition of collagenase mRNA expression, regardless of cell strain. The importance of fluid-flow-induced shear stress was confirmed when the additional boundary condition was predicted and showed that a decrease in shear stress with the same cell membrane strain resulted in less collagenase mRNA inhibition (3% strain at 6% strain per minute [C] versus 3% strain at 2% strain per minute [D]). However, the additional boundary condition also showed that increasing the membrane strain at similar low fluid-induced shear stress also resulted in a decrease in collagenase mRNA expression (1% strain at 2% strain per minute [A] versus 3% strain at 2% strain per minute [D]). Therefore, increased fluid-flow-induced shear stress alone and cell membrane strain alone were shown to inhibit the expression of rat interstitial collagenase mRNA. Thus, the results of this study confirm our hypothesis that fluid-flow-induced shear stresses and matrix-induced cell deformation are able to alter catabolic gene expression, presumably through a mechanotransduction pathway.

The interstitial fluid flow and extrusion out of tendon predicted by the model have been documented to occur in tendon following application of static and cyclic tensile loading (Hannafin and Arnoczky 1994; Helmer et al. 2006; Lanir et al. 1988) although the exact amount of fluid exuded per stretch remains unknown. The increased fluid flow predicted around the cell with increased loading rate may explain why collagenase mRNA expression decreases (Lavagnino et al. 2003). Similar to matrix-induced cell deformation, fluid flow has also been suggested to affect cell function and gene expression; however, the method of action has yet to be determined (Hannafin and

Arnoczky 1994). A previous study has shown that the fluid flow induced by cyclic or static load did not alter the diffusion of low molecular weight solutes compared to unloaded controls (Hannafin and Arnoczky 1994). These conclusions suggested that any biological benefit of cyclic loading (and resultant fluid flow) is probably not due to an increase in cell nutrition (Hannafin and Arnoczky 1994). Fluid flow, as suggested in bone, may alter gene expression as it induces a drag gradient on the pericellular matrix fibers (Han et al. 2004). The drag gradient on the pericellular fibers, which are connected to the cell membrane, in turn create a drag force on the cell, thereby creating strain amplification from fluid (Han et al. 2004). While the current model does not include pericellular matrix fibers, the increased fluid velocity with increased strain rate suggests that a similar phenomenon could occur in tendons.

An increase in fluid-induced shear stress on the cell could also explain the decreased collagenase expression with increased fluid flow. Fluid flow induces shear stress on the cell membrane and can mechanically stimulate cells to alter their gene expression (Archambault et al. 2002; Jin et al. 2000; Ng et al. 2005; You et al. 2000). The current model predicts that cyclic strain at low rate and amplitude results in an average shear stress of 0.157mPa. The magnitude of the shear stress increases significantly with increased rate (0.346mPa) or amplitude and rate (0.459mPa) corresponding with the inhibition of collagenase mRNA expression. While, the amount of fluid-induced shear stress on tendon cells under physiological conditions *in vivo* is unknown, both cartilage and tendon models have suggested that interstitial fluid flow may be in the range of 54nm/s to 10μm/s, resulting in fluid-induced shear of ~65mPa (Ateshian et al. 2007; Frank and Grodzinsky 1987; Ng et al. 2005). In a low flow

(6.3μm/s) study, myofibroblasts seeded in a 3D collagen gel scaffold were subjected to an estimated average fluid shear stress on the cells that varied between 15 and 33mPa, which were considered superphysiological (Ng et al. 2005). These values of shear stress from interstitial flow have been shown to strongly induce an anabolic response by fibroblasts (Ng et al. 2005). Therefore, the levels of fluid-induced shear stress predicted in this study appear to sufficiently approximate the values needed to inhibit catabolic gene expression. Previous studies have applied no load (stress-deprivation) (Arnoczky et al. 2004; Lavagnino et al. 2003) or much higher levels of fluid-induced shear stress to cells (0.1-2.5Pa) to induce a catabolic response (Archambault et al. 2002; Jin et al. 2000). Although suggested by the current study, additional studies are needed to determine whether a range of shear stress may exist that result in a homeostatic mechanobiological response, where an imbalance above or below that shear stress range would result in a catabolic response.

The matrix-induced cell deformation predicted by the model has also been documented to occur in tendon following application of static tensile load (Arnoczky et al. 2002; Hansen et al. 2002; Kastelic et al. 1980). In addition, static tendon load is known to correlate with nuclear strain (Arnoczky et al. 2002) and has been shown to have a dose-dependent effect on gene expression due to cytoskeletal deformation (Arnoczky et al. 2004). Cell deformation and the resulting alterations in the cytoskeleton are key components in the mechanotransduction response(s) of cells (Banes et al. 1995). The dose dependent inhibition of rat interstitial collagenase mRNA with cyclic strain amplitude shown in this study continues to support the mechanosensitive response of cells to deformation.

Therefore, both strain rate and strain amplitude appear to play a role in altering cellular gene expression in tendon. This mechanoresponsiveness of tendon cells is vital to maintaining tendon homeostasis (Lavagnino and Arnoczky 2005). The importance of both the fluid and solid components in transmitting mechanical signals to cells has been suggested in other biphasic tissue studies (Guilak and Mow 2000; Gupta and Haut Donahue 2006; You et al. 2000). Although this cellular response to global load has been studied in cartilage and meniscus (Guilak and Mow 2000; Gupta and Haut Donahue 2006), this is the first study to predict the mechanical environment of tendon cells to tensile loading.

Although the current study suggests that interstitial collagenase gene expression has both rate and amplitude dose dependence, it was not possible to determine whether rate or amplitude plays a more significant role in maintaining homeostasis. Studies in bone cells suggest fluid flow plays a significantly greater role than substrate deformation in activating gene expression (You et al. 2000). One reason for this dichotomy may be due to how fluid-shear and matrix strains play a role in mechanotransduction. With tendon cell deformation, calcium influx is a first and primary responder prior to alterations in gene expression (Archambault et al. 2002). However, even at high fluid-shear rates and with the induction of collagenase, there is no significant calcium influx in tendon cells (Archambault et al. 2002). Thus, gene expression may be activated through different mechanotransduction mechanisms (kinases, stretch-activated ion channels) depending on the mechanical signal experienced by the cell (Archambault et al. 2002). Indeed, kinases are cell membrane proteins that are phosphorylated when subjected to cyclic stretching or shear stress (Arnoczky et al. 2002; Iwasaki et al. 2000; Wang 2006).

A limitation of this study is that permeability is assumed constant throughout the tendon. Previous computational models and experiments have suggested that tendon permeability is anisotropic and strain dependent (Butler et al. 1997; Chen et al. 1998; Han et al. 2000; Yin and Elliott 2004; You et al. 2000). The use of constant permeability may limit the model from determining strain dependent changes in fluid flow that could help explain the weaker rate dependence of interstitial collagenase mRNA expression at higher strain amplitudes seen in this study. Future studies should investigate how the anisotropic, strain-dependent permeability of tendon would affect the magnitude of fluid flow around the cells.

Permeability was further investigated over the range of values seen in the literature (data not shown). The results of this analysis showed that the reduced permeability significantly lowered the fluid-flow induced shear stress predicted on the cell, while only modestly lowering the cell membrane strain. However, this change in permeability did not alter the overall relationship of shear stress between groups. The permeability of the PCM would also seem to have a potentially important impact on the shear stress developed on the cell membrane from fluid flow. Thus, this additional analysis demonstrates the importance of determining the material properties of tendon both at the global and cellular level to accurately model tendon mechanical signals on the cell.

The current model assumes that the collagen fibers act as springs and therefore the model does not allow for load transfer between fibers or between the fibers and the matrix and cell. Collagen crosslinking with both covalent (Haut 1985) and GAG bonds (Redaelli et al. 2003; Scott 2003) are thought to play an important role in tendon

mechanics. A recent study though, does not support the theory that sulfated GAG cross-links influence continuum-level mechanical behavior during quasi-static tensile loading (Lujan et al. 2007). However, the dermatin sulfate may still contribute to the viscoelastic response when other loading rates or protocols are used (Lujan et al. 2007). The fluid content of tendon determines the strain-rate-sensitive stiffness of tendon (Haut and Haut 1997). Therefore, although the crosslinking effect of GAGs may not be as important as previously theorized, the water content of tendon due to the GAG concentration may still contribute, to both the mechanical properties and the cell response of tendons. Therefore, although the collagen fibers as springs may be able to closely predict the global tendon response, they remain a limitation through their inability to transfer load to the matrix and thus alter the fluid response of the tendon upon loading.

The cellular stresses and strains in this study are only evaluated following the global applied displacement to peak strain in the first cycle. Therefore, the correlations between global loading, cell loading and the corresponding gene expression are all based on this one time point. The peak strain of the first cycle was assumed to validly represent the peak values of deformation and shear stress experienced by the cell. However, this assumption is another limitation of this study and additional studies are required to determine how tendon hysterisis and/or stress relaxation may affect mechanical signals at the cellular level over a 24-hour time period of cyclic strain.

In conclusion, the model was able to predict the global response of tendon to applied strain (stress, fluid exudation), as well as the associated cellular response of increased fluid-flow-induced shear stress with strain rate and matrix-induced cell deformation with strain amplitude. The model analysis, combined with the experimental

results, showed that both strain rate and strain amplitude are able to independently alter catabolic gene expression through increases in fluid-flow-induced shear stress and matrix-induced cell deformation respectively. Although shear stress and cell deformation appear to alter gene expression through mechanotransduction pathways, additional studies are required to separate the importance of these mechanical factors in homeostasis, injury, or rehabilitation.

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CONCLUSIONS

The goal of this dissertation was to investigate the role of cell mechanobiology in tendon health. The response of tendon cells to changing loading conditions has significant implications in unraveling the etiopathogenesis of tendinopathy (Arnoczky IJEP). This dissertation supports the hypothesis that the etiopathogenic stimulus for tendinopathy may occur from the mechanobiologic under-stimulation of tendon cells resulting from altered cell-matrix interactions.

Stress-deprivation of rat tail tendons results in the under-stimulation of tendon cells *in situ* and an immediate up-regulation of interstitial collagenease mRNA expression and protein synthesis [Appendix 1, Chapter 1] (Arnoczky et al. 2004; Lavagnino et al. 2003). Application of cyclic strain to tendons inhibits the up-regulation of this catabolic gene expression in a dose-dependent manner (both amplitude and frequency), presumably through a cytoskeletally based mechanotransduction pathway [Chapter 1] (Lavagnino et al. 2003). The applied strain rate and strain amplitude are able to independently alter interstitial collagenase gene expression through increases in fluid-flow-induced shear stress and matrix-induced cell deformation respectively [Chapter 5]. This ability of cyclic strain to inhibit interstitial collagenase, a degenerative enzyme involved in tendinopathy, may lead to advances in overuse injury prevention and optimal rehabilitation protocols following tendon injury and repair.

These results suggest that tendon cells may have a threshold, or set-point, with regard to their sensitivity to tensile loading and the resulting change in gene expression. Further investigation determined that tendon cells, when seeded into collagen gels, were able to demonstrate a mechanostat set point as changes in their cytoskeletal tension

(established or lost) corresponded to the alterations of anabolic (collagen) and catabolic (collagenase) gene expression in a reciprocal manner [Chapter 2] (Lavagnino and Arnoczky 2005). The long-term loss of cytoskeletal tension (21-day stress deprivation) in tendons results in a maintained up-regulation of interstitial collagenase, which, in turn, was shown to cause a significant loss of material properties of the tendon [Chapter 3] (Lavagnino et al. 2005). It was also shown that at high strains, isolated fibril damage could occur in tendons where the damaged fibril(s) are no longer able to transmit extracellular matrix loads to the tendon cells [Chapter 4] (Lavagnino et al. 2006). The localized stress-deprivation in this damaged area, will result in an under-stimulation of these cells [Chapter 1] (Lavagnino et al. 2003) which, initiates a catabolic response that can weaken the tendon [Chapter 3] (Lavagnino et al. 2005) making it more susceptible to damage from subsequent loading [Chapter 4] (Lavagnino et al. 2006). However the partially damaged tendon fascicle maintained its ability to bear load (Lavagnino et al. 2006). Therefore, it is possible that during a series of repetitive loading cycles a single abnormal loading cycle could produce strains sufficient enough to induce isolated fibril damage, but not cause clinical injury. Thus, while repetitive loading, per se, may not be responsible for initiating the cascade of events that lead to tendinopathy, it is likely that continued loading of the compromised tissue plays a significant role in the progression of the pathological process. Then when a critical level of damage has been reached the clinical and histological signs of tendinopathy may become evident (Arnoczky et al. 2007). Therefore, in conclusion this dissertation suggests that it is actually an absence of mechanical stimuli, secondary to microtrauma that is the mechanobiological stimulus for the degenerative cascade that leads to tendinopathy.

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APPENDIX A

Ex vivo static tensile loading inhibits MMP-1 expression in rat tail tendon cells through a cytoskeletally based mechanotransduction mechanism

Steven P. Arnoczky
Tao Tian
Michael Lavagnino
Keri L. Gardner

From the Laboratory for Comparative Orthopaedic Research
College of Veterinary Medicine, Michigan State University,
East Lansing, Michigan 48824, USA

Arnoczky, SP, Tian, T, Lavagnino, M, and Gardner, KL (2004) Ex vivo static tensile loading inhibits MMP-1 expression in rat tail tendon cells through a cytoskeletally based mechanotransduction mechanism J Ortho Res 22:328-333.

ABSTRACT

To determine the effect of various degrees of ex vivo static tensile loading on the expression of collagenase (MMP-1) in tendon cells, rat tail tendons were statically loaded in tension at 0.16, 0.77, 1.38 or 2.6 MPa for 24 h. Northern blot analysis was used to assay for mRNA expression of MMP-1 in freshly harvested, 24 h load deprived, and 24 h statically loaded tendons. Western blot analysis was used to assay for pro-MMP-1 and MMP-1 protein expression in fresh and 24 h load deprived tendons. Freshly harvested rat tail tendons demonstrated no evidence of MMP-1 mRNA expression and no evidence of the pro-MMP-1 or MMP-1 protein. Ex vivo load deprivation for 24 h resulted in a marked increase in the mRNA expression of MMP-1 which coincided with a marked increase of both pro-MMP-1 and MMP-1 protein expression. When tendons were subjected to ex vivo static tensile loading during the 24 h culture period, a significant inhibition of this upregulation of MMP-1 mRNA expression was found with increasing load ($p<0.05$). A strong ($r2=0.78$) and significant ($p<0.001$) inverse correlation existed between the level of static tensile load and the expression of MMP-1. Disruption of the actin cytoskeleton with cytochalasin D abolished the inhibitory effect of ex vivo static tensile loading on MMP-1 expression. The results of this study suggest that up-regulation of MMP-1 expression in tendon cells ex vivo can be inhibited by static tensile loading, presumably through a cytoskeletally based mechanotransduction pathway.

INTRODUCTION

The deleterious effects of immobilization on the structural and functional properties of ligaments and tendons are well-known [3,11,16,17,26-28]. While these alterations in the extracellular matrix appear to be cell mediated [17], the exact mechanism by which load affects tissue homeostasis is not completely understood. Previous work from our laboratory has demonstrated a progressive and significant decrease in the tensile modulus of load-deprived tendons in an in vitro system [17]. However, this effect was inhibited by application of low level (frequency and magnitude) cyclic strain [17]. Although the exact mechanism by which load deprivation decreases tensile modulus is still unclear, several investigators have implicated the role of interstitial collagenase (MMP-1) in this process [16,27]. A recent study has demonstrated that immobilization-induced up-regulation in the mRNA expression of MMP-1 in rabbit medial collateral ligaments could be inhibited by in vitro tensile loading [27]. Other in vitro studies have suggested that changes in cell shape can be correlated with a program of gene expression which is manifested by the degradation and synthesis of extracellular macromolecules [2,25,32,33]. Indeed, collagenase expression in fibroblasts has been shown to be up-regulated following chemically induced alterations in cell shape and a reorganization of the actin cytoskeleton [2,33].

A recent study has demonstrated that tensile loading of rat tail tendons results in an alteration of cell nuclear shape in a dose dependent manner [5]. Changes in cell and nuclear shape associated with changes in tendon tensile load are thought to be mediated through the extracellular matrix to the nucleus via transmembrane integrins and the cytoskeleton [8,15,31,34,35]. Thus, MMP-1 gene expression in tendons and ligament

cells could be influenced by mechanical load trhough such "mechanosensory" cytoskeletal tensegrity system(s). Thus, we hypothesized that MMP-1 gene expression in tendon cells varies with the magnitude of tensile load applied to the tendon and that this effect is mediated through the cytoskeleton.

MATERIALS AND METHODS

Tendons were obtained from the tails of adult Sprague Dawley rats. The tendons were removed immediately after euthanasia and maintained in Dulbecco's modified Eagle medium media supplemented with l0%, FBS, antibiotic/antimycotic solution, and ascorbate at 37 °C and l0% CO_2 for the duration of the experiments. Tendons were divided into the following groups: *Group 1*: freshly harvested tendons (time zero); *Group 2*: load deprived at 0 MPa for 24 h; *Group 3*: static tensile stress at 0.16 MPa for 24 h: *Group 4*: static tensile stress at 0.77 MPa for 24 h; *Group 5*: static tensile stress at 1.38 MPa for 24 h and *Group 6*: static tensile stress at 2.60 MPa for 24 h.

Static tensile load was applied by attaching clips (weighing 1.2 g) alone or clips with individual, calibrated stainless steel weights (5, 10, or 20 g) to an approximately 50 mm length of tendon (Figure 1). Stress values were calculated from representative microscopic measurements of the rat tail tendons (average diameter 320 μm). The weighted tendons were suspended in individual test tubes containing the above described media while load deprived tendons were placed in a 60 mm culture dish with the same media. All specimens were incubated for 24 h at 37 °C and 10% CO_2.

To determine the role of the cytoskeleton on the tensile load-modulated inhibition of MMP-1 mRNA expression, statically loaded (2.6 MPa) rat tail tendons were incubated in media containing 10 μM cytochalasin D (SIGMA, St Louis, MO).

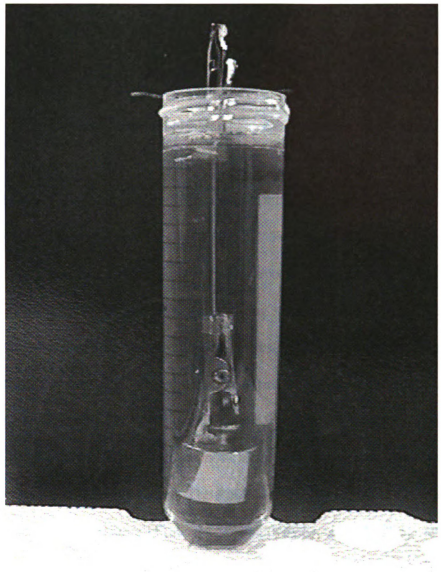


Figure A.1 Photograph of the static tensile loading system. Individual tendons were suspended in 50 ml test tubes filled with culture media containing 10% FBS and calibrated stainless steel weights were attached by clips.

Construction of rat MMP-13 DNA plasmid: To prepare the probe, rat MMP-1 DNA (GeneBank M60616) was amplified by polymerase chain reaction using rat genomic DNA template. The oligonucleotide primers used were 5'-GCC CAT ACA GTT TGA ATA CAG TAT CTG-3' and 5'-CCA GTT TAA TAA ACA CCA TCT CTT GA-

3'. PCR product (1167-bp) was subjected to electrophoresis on 1% agarose gel and recovered using Qiaex II Kit (QIAGEN Inc., Valencia. CA), and then cloned into PCR ®2.l-TOPO® plasmid using TOPO TA cloning kit (Invitrogen Living Science, Carlsbad, CA). The plasmid was transformed into competent *E. coli* with selection for kanamvcin and ampicillin resistance. The construction was confirmed by restriction enzyme digestion (ECOR 1, EcoR V and Hind III) and polymerase chain reaction.

RNA extraction und northern blot analysis: Twenty rat tail tendons per group per experiment were used, and the experiment was repeated three times. At the end of the experimental period an approximately 40 mm long segment (which did not include any portion of the tendon in contact with the clips used to attach the weight or to suspend the tendon) of the statically loaded tendons and the entire length of the load deprived tendons were collected. Total cellular RNA was extracted from the 20 tendons from each group by the acid guanidine thiocyanate-phenol-chloroform procedure (totally RNA kit, Ambion Inc., Austin, TX) and pooled. The RNA samples were loaded (8 μg/lane) onto a 1.2% agarose gel containing 0.66 M formaldehyde and MOPS, subjected to electrophoresis, and transferred to a nylon membrane (Pierce Corp. Rockford. IL) for 1 h in TAE at 300 mA. Following transfer, the membrane was air-dried and UV cross-linked at 10 J/cm^2.

The RNA blots were hybridized with a DNA probe for rat MMP-1 mRNA generated in our lab and a human G3PDH cDNA control probe (Clontech Laboratories, Inc. Palo Alto, CA). The probes were labeled with biotin using the North2South Direct HRP Labeling and Detection Kit (Pierce Corp, Rockford, IL). The RNA blots were hybridized with labeled probes (10 ng/ml hybridization solution) at 55 °C for 1 h and

then washed. The membrane was then incubated with a chemiluminescent working solution for 5 min and exposed to films for 5-10 min. The films were scanned with a laser film digitizer (Lumiscan 75, Lumisys, Inc., Sunnyvale, CA), and MMP-1 mRNA expression was quantified by optical density measurements using Scion Image (Scion Corporation, Frederick, MD) as a ratio of G3PDH expression. The effect of load magnitude was evaluated using a Kruskal-Wallis test, followed by a Mann-Whitney U post hoc test, and the correlation between tensile load and mRNA expression of MMP-1 was examined using a polynomial regression analysis ($f = 1/a + bx$). Significance was set at $p<0.05$.

Western blot analysis: To verify that MMP-1 mRNA expression coincided with the expression of the MMP-1 protein, rat tail tendons (freshly harvested [$n = 20$] or load-deprived for 24 h [$n = 20$] as noted above) were processed for Western immunoblot staining. Total protein was extracted from each group of pooled tendons by homogenizing the tendons and lysing the cells overnight with a buffer containing 25 mM HEPES, 0.5 mM Na_3VO_4, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1% Triton, 0.1 mM PMSF, 1 μg aprotinin, 1 μM microcystin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. The lysates were centrifuged (10^4 RPM at 4 °C for 30 min), and the protein quantified (BioRad Protein Assay. BioRad Labs, Hercules, CA) and electrophoresed on a 12.5% SDS-polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane and analyzed for pro-MMP-1 protein and MMP-1 protein using a mouse monoclonal antibody (Oncogene, Boston, MA) and chemiluminescence detection (New

England BioLabs, Beverly, MA). Human MMP-1 protein (Sigma, St. Louis, MO) was used as the positive control.

Actin staining/confocal laser microscopy: To verify in situ disruption of the actin cytoskeleton by cytochalasin D, additional rat tail tendons were prepared for actin staining and confocal laser microscopy. Ten millimeter segments of control (n = 5) and experimental (n = 5 treated for 1 h with 10 μM cytochalasin D) rat tail tendons were fixed in 10% phosphate buffered formalin and stained with rhodamine phalloidin (5 units/ml) (Molecular Probes, Eugene, Oregon). The tendons were wet mounted on a glass slide for viewing with a Zeiss Pascal Laser Scanning Confocal microscope (Carl Zeiss, Thornwood. NY). Observations were made using a 40x oil immersion objective with a coverslip. Fluorescent images were obtained using a HeNe 543 nm laser with a long pass 560 nm emission filter. After localizing a representative cell, a 2x zoom was used to obtain the image. Overlay images were achieved by multiple confocal 0.6 μm thick 'z-sectioning' through the depth of the cell and then combining the images into a single overlay image.

Drugs and Chemicals: Except as noted, all drugs and chemicals (Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), ascorbate, gentamicin, and penicillin/streptomycin-fungizone solution) were obtained from Gibco, Grand Island, New York.

RESULTS

The yield of total RNA extracted from each group was 0.2 μg/mg wet weight and was not significantly different among groups.

Freshly harvested rat tail tendons *(Group 1)* demonstrated no appreciable MMP-1 mRNA expression on Northern blots. Tendons in the load-deprived group *(Group 2)* cultured for 24 h demonstrated substantial mRNA expression of MMP-1 (Figure 2). When tendons were subjected to static tensile loading during the same 24 h culture period, MMP-1 mRNA expression decreased significantly with increasing load ($p < 0.05$) (Figure 2 and Table 1).

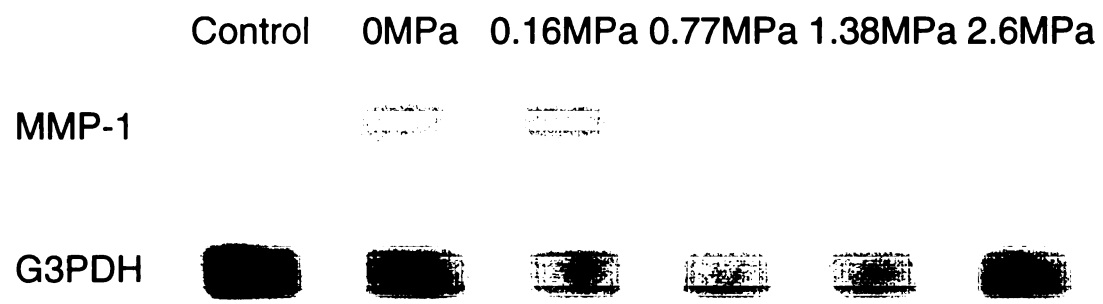


Figure A.2 Representative Northern blot gel illustrating the relative expression of MMP-1 mRNA as a function of applied stress for 24 h. G3PDH was used as an internal control. Experiments were performed three times and a representative result is shown.

Table A.1 The effect of static stress on MMP-1 expression

STRESS	MMP-1/G3PDH
Fresh tendons[a]	0^{bcdef}
0.00 MPa for 24 h[b]	0.670 ± 0.266^{adef}
0.16 MPa for 24 h[c]	0.680 ± 0.088^{aef}
0.77 MPa for 24 h[d]	0.333 ± 0.153^{ab}
1.38 MPa for 24 h[e]	0.116 ± 0.050^{abc}
2.60 MPa for 24 h[f]	0.077 ± 0.017^{abc}
Superscripts a-e indicate significantly different corresponding data pairs ($p < 0.05$).	

A strong (r^2 = 0.78) and significant ($p < 0.001$) inverse correlation was found between the level of static tensile load and the expression of MMP-1 mRNA (Figure 3). However, MMP-1 mRNA expression was not completely abolished at the highest stress (2.6 MPa) examined.

Western immunoblot staining revealed no appreciable pro-MMP-1 or MMP-1 protein expression in the freshly harvested rat tail tendons. However, following 24 h of load deprivation, substantial pro-MMP-1 and MMP-1 protein expression was detected in the tendons (Figure 4).

Treatment with cytochalasin D did not influence MMP-1 mRNA expression in the load deprived tendons *(Group 2)* ($p > 0.05$). However, cytochalasin D treatment did significantly ($p< 0.05$) increase MMP-1 mRNA expression in tendons exposed to a tensile loading stress of 2.60 MPa (Figure 5).

Actin staining and confocal laser microscopic examination demonstrated the presence of actin stress filaments within the cells of the time zero control tendons (Figure 6A). However, in those tendons incubated for 1 h in 10 μM cytochalasin D, the actin appeared globular, and no evidence of organized actin stress filaments was found (Figure 6B).

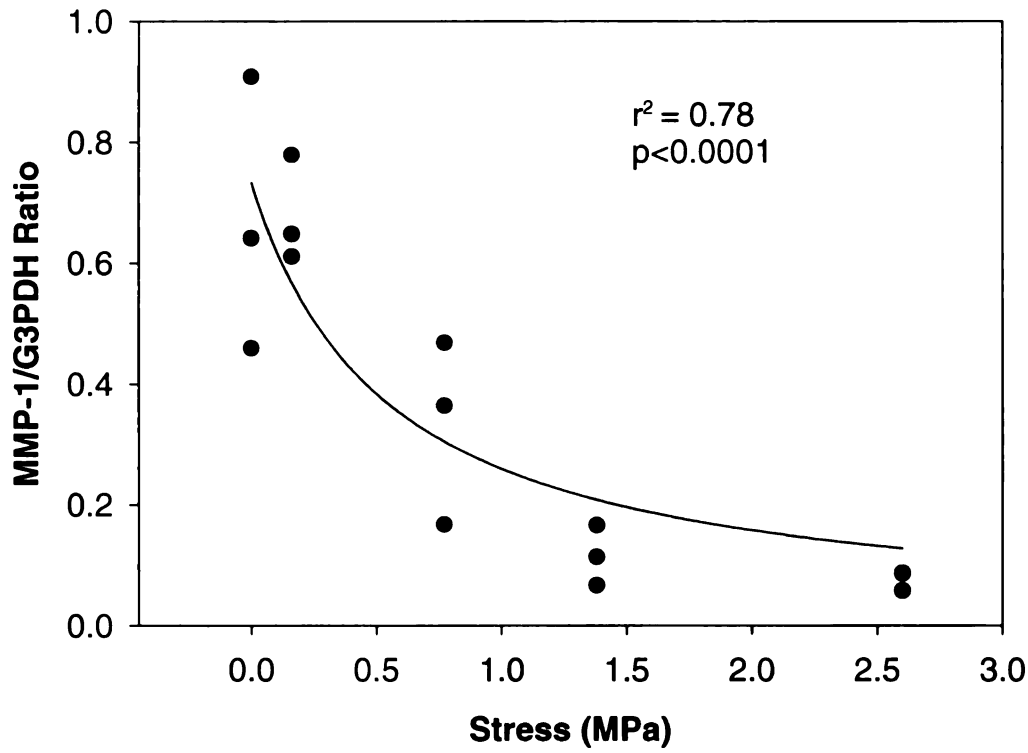


Figure A.3 Composite polynomial regression of graph of the three experimental replicates plotting MMP-1 mRNA expression (normalized as a ratio of MMP-1 to G3PDH) against static stress. There was a strong (r^2 = 0.78) and significant (p < 0.0001) inverse correlation between MMP-1 mRNA expression and static stress.

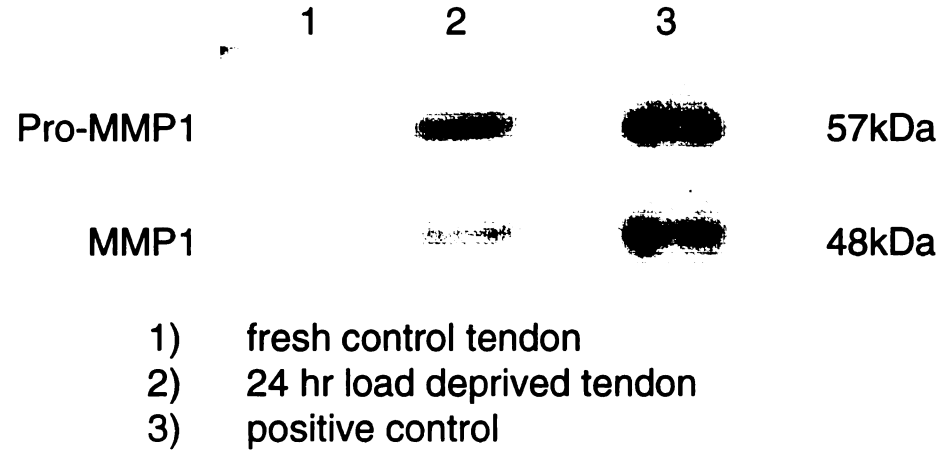
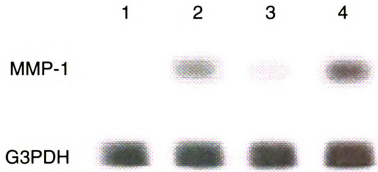


Figure A.4 Western blot analysis illustrating the absence of pro-MMP-1 and MMP-1 protein expression in freshly harvested rat tail tendons. There was a significant up-regulation of pro-MMP-1 and MMP-1 protein expression in the tendon cells after 24 h of in vitro load deprivation.



1) Fresh Control
2) 24 hr 0MPa Static Tensile Stress
3) 24 hr 2.6MPa Static Tensile Stress
4) 24 hr 2.6MPa Static Tensile Stress in 10μM cytochalasin

Figure A.5 Representative Northern blot gel illustrating the relative expression of MMP-1 mRNA in fresh control tendons (lane 1): 24 h load deprived at 0 MPa (lane 2); 24 h at 2.60 MPa static tensile stress (lane 3): 24 h at 2.60 MPa static tensile stress in 10 μM cytochalasin D (lane 4). G3PDH was used as an internal control. Experiments were performed three times and a representative result is shown.

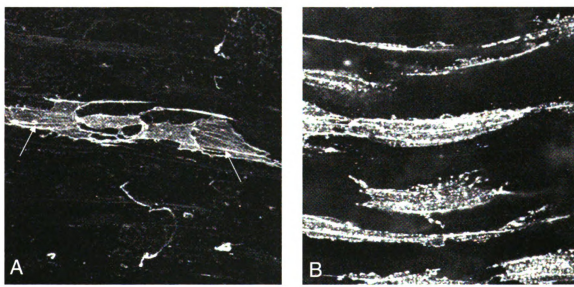


Figure A.6 Confocal overlay images of rat tail tendon cells stained with rhodamine phalloidin to label actin filaments. (A) Fresh control tendon: note presence of actin stress fibers (arrows) in cytoskeleton. (B) Tendon treated with 10 μM cytochalasin D for 1 h; note the absence of organized actin stress fibers. (confocal 40x oil immersion: 2x zoom).

DISCUSSION

The strain-induced signaling of cells through mechanotransduction pathways is thought to play a significant role in maintaining the normal homeostasis of connective tissues [1,8,31,34,35]. While various stress levels have been shown to induce anabolic responses in bone [12,21,22,29,30], ligament [9,20,23], and tendon cells [6,7], stress deprivation has been associated with tissue catabolism [1,3,10,16,17,30]. These catabolic changes have been attributed to degradation of the extracellular matrix by matrix metalloproteinases (MMPs) [3,16,27]. The results of the current study demonstrate that, when compared to freshly harvested control tendons, ex vivo load deprivation results in an immediate (within 24 h) increase in MMP-1 mRNA expression and MMP-1 protein expression in the tendon cells. While in vivo studies have reported similar findings in immobilized ligaments and tendons [16,27], the precise mechanism by which load deprivation triggers MMP-1 gene expression in these tissues is unclear.

A proposed mechanism of mechanotransduction theorizes that extracellular matrix strain imparts a strain to cells through their integrin based cell-matrix connections [8,31,34,35]. The resulting cellular deformation creates changes in tension within the cytoskeleton, which can be sensed by the cell nucleus through a mechanosensory tensegrity system and results in specific cellular responses [8]. While most studies have focused on the effect of increased (over normal resting levels) strain on the mechanotransduction signaling responses of tendon cells in monolayer [6,7], the impact of total load deprivation on this process has not been examined in whole tendons ex vivo. A recent study suggested that cells are capable of establishing a constant level of tension between themselves and their extracellular matrix through a cytoskeletal tensegrity

system [13]. This tensional homeostasis is thought play an important role in the mechanotransduction response of the cell [13]. Removal of tendons from their normal mechanical environment could significantly alter this homeostatic tension within the cytoskeletal tensegrity system and be responsible for up-regulation of MMP-13 mRNA expression seen following 24 h of load deprivation.

An in vitro study has shown that MMP-1 expression in fibroblasts is significantly higher in free (contracting) gels than in anchored gels [25]. This suggests that the mechanical forces that are generated by the cell between itself and its surrounding matrix may, in part, regulate MMP-1 expression. Sustained loss of normal cell-matrix tension, as could occur when tendons have been removed from their origins and insertions and placed in load deprived environment for 24 h, could have played a role in triggering the MMP-1 expression seen in this study.

Changes in cell shape, specifically the loss of actin stress fiber organization, has been strongly correlated with collagenase gene expression [2,33]. It has been shown that procollagenase expression in rabbit synovial fibroblasts was only induced by treatments that modified cellular actin [2,33]. This would suggest that a change in the state of actin assembly may influence collagenase expression. In the current study, treatment with cytochalasin D, a fungal product which depolymerizes actin filaments, completely abrogated the load induced inhibition of MMP-1 mRNA expression. This further supports the role of a cytoskeletally based mechanosensory tensegrity system in the control of MMP-1 mRNA expression in tendon cells.

The application of a tensile load to the tendons in culture resulted in a decrease in MMP-1 mRNA expression with increasing load. While MMP-1 mRNA expression ex

vivo was decreased by increasing load, this inhibitory effect was non-linear and was not completely eliminated at the loads examined. A plausible explanation for this observation is the non-linear stress-strain behavior exhibited by rat tail tendons [18]. It is likely that with increasing load an increasing number of cells undergo nuclear (and presumably cytoskeletal) deformation [5]. Thus, the increase in the number of cells receiving a mechanotransduction stimulus with increasing load may reflect the increased inhibition of MMP-1 mRNA expression. The inability to completely eliminate MMP-1 mRNA expression at the loads sampled could be explained by the fact that the loads examined in this study were all within the reported "toe" region (<3% strain) of the stress-strain curve for rat tail tendon fascicles [18]. A recent study has shown that collagen crimp within the center of the fascicles might not be completely eliminated until 2.8% strain [18]. This could mean that, in the current study, cells within the center of the fascicle were not exposed to stresses sufficient to activate mechanotranduction responses that could affect (inhibit) MMP- 1 mRNA expression.

Finally, the exact influence of an ex vivo environment on the expression of MMP-1 mRNA in rat tail tendon cells is unknown. While the experimental design of this study would suggest that under similar ex vivo conditions static tensile loading does inhibit the expression of MMP-1 mRNA when compared to load-deprived tendons, the incomplete inhibition of this expression with what would appear to be physiologic loads may also be related to the ex vivo environment. A recent review article suggests that the absence of innervation and vascularization in explant culture systems may also contribute to the regulation of specific gene expression in connective tissues [19]. Thus, the interpretation of mechanotransduction-induced results from such culture systems must be tempered

with the possibility that the gene response seen ex vivo may not be reflective of that which occurs in vivo [19]. However, the effect of rigorously controlled loading and load deprivation conditions on MMP-1 mRNA expression in tendon cells ex vivo seen in the current study are similar to those reported for in vivo immobilized ligaments and tendons [16,27] and in vitro cyclically loaded ligaments [27] and fibrochondrocytes [1]. A comparison of these results would suggest that the mechanostimulation of tendon cells (whether in vivo or ex vivo) does play a significant role in the regulation of MMP-1 mRNA expression.

The results obtained from this ex vivo test system demonstrate that MMP-1 mRNA expression in tendon cells can be affected by static tensile load, presumably through a cytoskeletally based mechanotransduction pathway. MMP-1 was chosen as the gene of interest based on a previous in vitro study that documented an increase in MMP-1 mRNA expression and not MMP-13 mRNA following immobilization [27]. While this study only examined the effects of various levels of static tensile load on this signaling system, cyclic loading (which reflects a more relevant in vivo loading environment) and the resulting fluid flow have also been shown to play a key role in the mechanotransduction response of connective tissues [4,14,23,24]. This is especially true in bone where fluid flow is believed to be an important physical signal that influences bone cell metabolism and bone adaptation to mechanical loading [14,24,30]. The effect of cyclic load and fluid flow on the cytoskeletal components of the mechanosensory tensegrity system of tendon cells must also be investigated to completely understand the interaction of mechanical loading and tendon physiology. As in bone [21], the effect of

loading history on cytoskeletal adaptations and matrix interactions could provide a key to understanding the effect of various mechanical loading regimes on tendon health.

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APPENDIX B

B.1 Global Model for 1% strain at 2% strain/minute

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*Heading
*Node
      1, 0., 0.
      2, 0.05, 0.
      3, 0.1, 0.
      4, 0.15, 0.
      5, 0., 0.2
      6, 0.05, 0.2
      7, 0.1, 0.2
      8, 0.15, 0.2
      9, 0., 0.4
     10, 0.05, 0.4
     11, 0.1, 0.4
     12, 0.15, 0.4
     13, 0., 0.6
     14, 0.05, 0.6
     15, 0.1, 0.6
     16, 0.15, 0.6
     17, 0., 0.8
     18, 0.05, 0.8
     19, 0.1, 0.8
     20, 0.15, 0.8
     21, 0., 1.
     22, 0.05, 1.
     23, 0.1, 1.
     24, 0.15, 1.
     25, 0., 1.2
     26, 0.05, 1.2
     27, 0.1, 1.2
     28, 0.15, 1.2
     29, 0., 1.4
     30, 0.05, 1.4
     31, 0.1, 1.4
     32, 0.15, 1.4
     33, 0., 1.6
     34, 0.05, 1.6
     35, 0.1, 1.6
     36, 0.15, 1.6
     37, 0., 1.8
     38, 0.05, 1.8
     39, 0.1, 1.8
     40, 0.15, 1.8
     41, 0., 2.
     42, 0.05, 2.
     43, 0.1, 2.
     44, 0.15, 2.
     45, 0., 2.2
     46, 0.05, 2.2
     47, 0.1, 2.2
     48, 0.15, 2.2
     49, 0., 2.4
     50, 0.05, 2.4
     51, 0.1, 2.4
     52, 0.15, 2.4
     53, 0., 2.6
     54, 0.05, 2.6
     55, 0.1, 2.6
     56, 0.15, 2.6
     57, 0., 2.8
     58, 0.05, 2.8
     59, 0.1, 2.8
     60, 0.15, 2.8
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     62, 0.05, 3.
     63, 0.1, 3.
     64, 0.15, 3.
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     79, 0.1, 3.8
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*Element, type=SPRINGA,
elset=MedHigh
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306, 6, 10
310, 10, 14
314, 14, 18
318, 18, 22
322, 22, 26
326, 26, 30
330, 30, 34
334, 34, 38
338, 38, 42
342, 42, 46
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*Element, type=SPRINGA,
elset=MedLow
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307, 7, 11
311, 11, 15
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319, 19, 23
323, 23, 27
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695, 395, 399
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*Element, type=SPRINGA,
elset=Low
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668, 368, 372
672, 372, 376
676, 376, 380
680, 380, 384
684, 384, 388
688, 388, 392
692, 392, 396
696, 396, 400
700, 400, 404
*Spring, elset=High,
nonlinear

0, 0
0, 0.0044
400, 20
*Spring, elset=MedHigh,
nonlinear

0, 0
0, 0.004
400, 20
*Spring, elset=MedLow,
nonlinear

0, 0
0, 0.0032
400, 20
*Spring, elset=Low,
nonlinear

0,0
0, 0.0006
400, 20
*Orientation, name=Ori-
1
          1., 0., 0.,
0., 1., 0.
2, 0.
** Region: (Section-
1:Picked), (Material
Orientation:Picked)
*Elset, elset=_I1,
generate
   1,  300,    1
** Section: Section-1
*Solid Section,
elset=_I1,
orientation=Ori-1,
material=YINmatrix
1.,
*Nset,
nset=_PickedSet24,
generate
   4,  404,    4
*Elset,
elset=_PickedSet24,
generate
   3,  300,    3
*Nset,
nset=_PickedSet26,
generate
 401,  404,    1
*Elset,
elset=_PickedSet26,
generate
 298,  300,    1
*Nset, nset=NALL,
generate
   1,  404,    1
*Elset, elset=NALL,
generate
   1,  300,    1
*Nset,
nset=_PickedSet34,
generate
 1,  4,  1
```

```
*Elset,
elset=_PickedSet34,
generate
 1,  3,  1
*Nset, nset=_halfLine,
generate
 201,  204,    1
**
** MATERIALS
**
*Material,
name=YINmatrix
*Elastic,
type=ENGINEERING
CONSTANTS
 0.0457, 1., 0.0457,
0.07769, 0.7, 1.7, 0.1,
0.013441, 0.1
*Permeability,
specific=9.81e-06
 3.1392e-09,2.
** BOUNDARY CONDITIONS
** Name: Free Edge
Type: Pore pressure
*Boundary
_PickedSet24, 8, 8
** Name: Radial Type:
Displacement/Rotation
*Boundary
_PickedSet34, 2, 2
*Boundary
NALL, 3, 3
*INITIAL CONDITIONS,
TYPE=RATIO
NALL, 2.
** ----------------------
** 1% strain at 2%
strain/minute
** STEP: Step-1
**
*Step, name=Step-1,
nlgeom, amplitude=RAMP,
inc=1000
*Soils, consolidation,
end=PERIOD
1., 30., , ,
*El Print, elset=_I1,
SUMMARY=YES
 COORD, FLVEL
*CONTROLS,
PARAMETERS=FIELD,
FIELD=PORE FLUID
PRESSURE
,,6.329E-06
** BOUNDARY CONDITIONS
** Name: Displacement
Type:
Displacement/Rotation
*Boundary
_PickedSet26, 2, 2, 0.2
** ----------------------
** OUTPUT REQUESTS
*Restart, write,
frequency=1
**
** FIELD OUTPUT: F-
Output-1
**
*Output, field
*Node Output
U, RF, POR
*Element Output
S, LE, VOIDR, SAT,
FLVEL
**
** HISTORY OUTPUT: H-
Output-1
**
*Output, history,
variable=PRESELECT
*El Print, freq=999999
*Node Print,
freq=999999
*FILE FORMAT, ZERO
INCREMENT
*End Step
```

B.2 Global Model for 1% strain at 20% strain/min

```
** ----------------------
** 1% strain at 20%
strain/minute
** STEP: Step-1
*Step, name=Step-1,
nlgeom, amplitude=RAMP,
inc=1000
*Soils, consolidation,
end=PERIOD
0.1, 3., , ,
*CONTROLS,
PARAMETERS=FIELD,
FIELD=PORE FLUID
PRESSURE
,,6.329E-06
** BOUNDARY CONDITIONS
** Name: Displacement
Type:
Displacement/Rotation
*Boundary
_PickedSet26, 2, 2, 0.2
** ----------------------
```

B.3 Global Model for 3% strain at 6% strain/minute

```
** ----------------------
** 3% strain at 6%
strain/minute
** STEP: Step-1
*Step, name=Step-1,
nlgeom, amplitude=RAMP,
inc=1000
*Soils, consolidation,
end=PERIOD
1., 30., , ,
*CONTROLS,
PARAMETERS=FIELD,
FIELD=PORE FLUID
PRESSURE
,,6.329E-06
** BOUNDARY CONDITIONS
** Name: Displacement
Type:
Displacement/Rotation
*Boundary
_PickedSet26, 2, 2, 0.6
** ----------------------
```

B.4 Global Model for 3% strain at 2% strain/minute

```
** ------------------
** 3% strain at 2%
strain/minute
** STEP: Step-1
*Step, name=Step-1,
nlgeom, amplitude=RAMP,
inc=1000
*Soils, consolidation,
end=PERIOD
1., 130., , ,
*CONTROLS,
PARAMETERS=FIELD,
FIELD=PORE FLUID
PRESSURE
,,6.329E-06
** BOUNDARY CONDITIONS
** Name: Displacement
Type:
Displacement/Rotation
*Boundary
_PickedSet26, 2, 2, 0.6
** ------------------
```

B.5 Submodel for 1% strain at 2% strain/minute

```
*Heading
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2, 0.1225, 9.89
3, 0.1225, 9.91
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5, 0.13, 9.89
6, 0.1275, 9.89
7, 0.1275, 9.91
8, 0.13, 9.91
9, 0.127, 9.91
10, 0.123, 9.91
11, 0.123, 9.89
12, 0.127, 9.89
13, 0.1, 9.8
14, 0.15, 9.8
15, 0.15, 10.
16, 0.1, 10.
17, 0.1273756, 9.889221
18, 0.1270097, 9.888513
19, 0.1264553, 9.887967
20, 0.125782, 9.887626
21, 0.1250419, 9.887501
22, 0.1243278, 9.887592
23, 0.1237166, 9.887855
24, 0.123218, 9.888247
25, 0.1228318, 9.888756
26, 0.1225847, 9.889355
27, 0.1225, 9.891253
28, 0.1225, 9.892502
29, 0.1225, 9.893751
30, 0.1225, 9.895
31, 0.1225, 9.89625
32, 0.1225, 9.8975
33, 0.1225, 9.89875
34, 0.1225, 9.9
35, 0.1225, 9.90125
36, 0.1225, 9.9025
37, 0.1225, 9.90375
38, 0.1225, 9.904999
39, 0.1225, 9.906248
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42, 0.122596, 9.910686
43, 0.1228836, 9.911331
44, 0.1233149, 9.911847
45, 0.1238201, 9.912204
46, 0.1245113, 9.912452
47, 0.1252149, 9.912491
48, 0.1259368, 9.912318
49, 0.1265545, 9.911958
50, 0.1270634, 9.911411
51, 0.1273919, 9.910728
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1101,561,1171,1181,562
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1103,1172,1173,1183,1182
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1122,1190,238,239,1200
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1124,1191,1192,1202,1201
1125,1192,1193,1203,1202
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1138,1204,1205,246,247
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1141,1207,1208,243,244
1142,1208,1209,242,243
1143,1209,1210,241,242
1144,1210,240,16,241
*Element, type=SPRINGA,
Elset=MedLow
1145, 13, 16
*Element, type=SPRINGA,
Elset=Low
1146, 14, 15
*Spring, Elset=MedLow,
nonlinear

0, 0
0, 0.0032
400, 20
*Spring, Elset=Low,
nonlinear

0,0
0, 0.0006
400, 20
*Orientation,name=Ori-3
          1., 0., 0.,
0., 1., 0.
2, 0.
** Region: (PCM-
Section:Picked),
(Material
Orientation:Picked)
*Elset, elset=_I1,
generate
   1,  162,    1
** Section: PCM-Section
*Solid Section,
elset=_I1,
orientation=Ori-3,
material=YINPCM
1.,
** Region: (Cell-
Section:Picked),
```

```
(Material
Orientation:Picked)
*Elset, elset=_I2,
generate
 163,  288,    1
** Section: Cell-
Section
*Solid Section,
elset=_I2,
orientation=Ori-3,
material=YINCELL
1.,
** Region: (membrane-
section:Picked),
(Material
Orientation:Picked)
*Elset, elset=_I3,
generate
 289,  342,    1
** Section: membrane-
section
*Solid Section,
elset=_I3,
orientation=Ori-3,
material=YINMEM
1.,
** Region: (ECM-
Section:Picked),
(Material
Orientation:Picked)
*Elset, elset=_I4,
generate
  343,  1144,    1
** Section: ECM-Section
*Solid Section,
elset=_I4,
orientation=Ori-3,
material=YINMatrix
1.,
*Elset,
elset=shearstress
289, 290, 291, 292,
293, 294, 295, 296,
297, 298, 299, 300,
301, 302, 303, 304
305, 306, 307, 308,
309, 310, 311, 312,
313, 314, 315, 316,
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328, 329, 330, 331,
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6,   9,  12,  15,  18,
21,  24,  27, 30,  33,
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51,  54,  57,  60,  63,
64,  67,  70,  73,  76,
79,  82,  85,  88,  91,
94,  97, 100, 105, 108,
111, 114, 117, 120,
123, 126, 129, 132,
135, 138, 141, 144,
147, 150, 153, 156,
159, 162
*Nset, nset=NSUB,
generate
    1,  1210,    1
*Elset, elset=NSUB,
generate
    1,  1144,    1
*Nset, nset=_bottom,
generate
 13,  14,    1
*Nset,
nset=_PickedSet76
  13,  14,  15,  16,
167, 168, 169, 170,
171, 172, 173, 174,
175, 176, 177, 178
 179, 180, 181, 182,
183, 184, 185, 186,
187, 188, 189, 190,
191, 192, 193, 194
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271, 272, 273, 274
 275, 276, 277, 278,
279, 280, 281, 282,
283, 284, 285, 286,
287, 288, 289, 290,
291, 292
*Elset,
elset=_PickedSet76
343,  344,  345,  346,
347,  348,  349,  350,
351,  352,  353,  364,
375,  386,  397,  408,
419,  430,  441,  442,
443,  444,  445,  446,
447,  448,  449,  450,
451,  452,  453,  454,
455,  505,  507,  509,
520,  523,  526,  529,
530,  531,  532,  564,
572,  580,  588,  596,
604,  612,  620,  628,
636,  644,  652,  660,
668,  676,  684,  692,
700,  708,  716,  724,
732,  740,  748,  756,
764,  772,  780,  788,
796,  804,  812,  854,
857,  860,  863,  866,
869,  872,  875,  876,
904,  905,  906,  907,
908,  909,  910,  990,
991,  992,  993,  994,
995,  996,  997,  998,
999, 1000, 1001, 1012,
1023, 1034, 1045, 1056,
1067, 1078, 1089, 1100,
1111, 1122, 1133, 1134,
1135, 1136, 1137, 1138,
1139, 1140, 1141, 1142,
1143, 1144
*Submodel,
exteriorTolerance=0.01
_PickedSet76,
**
** MATERIALS
*Material, name=YINCELL
*Elastic
 0.5, 0.069
*Permeability,
specific=9.81e-06
 4.5e-09,2.
*Material, name=YINMEM
*Elastic
1., 0.49
*Permeability,
specific=9.81e-06
 5e-14,2.
*Material,
name=YINMatrix
*Elastic,
type=ENGINEERING
CONSTANTS
0.0457, 1., 0.0457,
0.07769, 0.7, 1.7,
0.5, 0.013441, 0.5,
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*Permeability,
specific=9.81e-06
 3.1392e-09,2.
*Material, name=YINPCM
*Elastic
1., 0.49
*Permeability,
specific=9.81e-06
 4e-09,2.
*Material,
name=membrane
*Elastic,
type=ENGINEERING
CONSTANTS
1.,    70.,    1.,
0.01,   0.3,   0.7,
5., 0.385, 5.,
*Permeability,
specific=9.81e-06
 9.81e-08,2.
*INITIAL CONDITIONS,
TYPE=RATIO
NSUB, 2.
*Boundary
_bottom, 3, 3
** ----------------------
** ----------------------
** 1% strain at 2%
strain/minute
** STEP: Step-1
**
*Step, name=Step-1,
nlgeom, amplitude=RAMP,
inc=1000
*Soils, consolidation,
end=PERIOD
5., 30., , ,
*El Print,
elset=shearstress,
FREQUENCY=30
 COORD, FLVEL
*CONTROLS,
PARAMETERS=FIELD,
FIELD=PORE FLUID
PRESSURE
,,0.001
** ----------------------
**----------------------
**
** BOUNDARY CONDITIONS
**
** Name: Sub Type:
Submodel
*Boundary, submodel,
step=1, timescale
_PickedSet76, 1, 1
_PickedSet76, 2, 2
_PickedSet76, 8, 8
**
** OUTPUT REQUESTS
**
*Restart, write,
frequency=1
**
** FIELD OUTPUT: F-
Output-1
**
*Output, field
*Node Output
U, RF, POR
*Element Output
S, LE, VOIDR, SAT,
FLVEL, COORD
**
** HISTORY OUTPUT: H-
Output-1
**
*Output, history,
variable=PRESELECT
*El Print, freq=999999
*Node Print,
freq=999999
*FILE FORMAT, ZERO
INCREMENT
*End Step
```

B.6 Submodel for 1% strain at 20% strain/min

```
** ----------------------
** ----------------------
**
** STEP: Step-1
**
*Step, name=Step-1,
nlgeom, amplitude=RAMP,
inc=1000
*Soils, consolidation,
end=PERIOD
0.1, 3., , ,
*El Print,
elset=shearstress,
FREQUENCY=30
 COORD, FLVEL
*CONTROLS,
PARAMETERS=FIELD,
FIELD=PORE FLUID
PRESSURE
,,0.001
** ----------------------
** ----------------------
```

B.7 Submodel for 3% strain at 6% strain/minute

```
** ----------------------
** ----------------------
**
** STEP: Step-1
**
*Step, name=Step-1,
nlgeom, amplitude=RAMP,
inc=1000
*Soils, consolidation,
end=PERIOD
1., 30., , ,
*El Print,
elset=shearstress,
FREQUENCY=30
 COORD, FLVEL
*CONTROLS,
PARAMETERS=FIELD,
FIELD=PORE FLUID
PRESSURE
,,0.001
** ----------------------
** ----------------------
```

B.8 Submodel for 3% strain at 2% strain/minute

```
** ----------------------
** ----------------------
**
** STEP: Step-1
**
*Step, name=Step-1,
nlgeom, amplitude=RAMP,
inc=1000
*Soils, consolidation,
end=PERIOD
1., 130., , ,
*El Print,
elset=shearstress,
FREQUENCY=30
 COORD, FLVEL
*CONTROLS,
PARAMETERS=FIELD,
FIELD=PORE FLUID
PRESSURE
,,0.001
** ----------------------
** ----------------------
```